Development of advanced chromatographic techniques for the in-depth phenolic profiling of rooibos

by

Nico Albertus Walters

Thesis presented in partial fulfilment of the requirements for the degree of **Master of Science in Food Science in the Faculty of AgriSciences at Stellenbosch University**

Study leader: Prof. D. de Beer

Co-study leaders: Prof. A.J. de Villiers and

Dr. P.J. Williams

December 2016

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2016

Summary

Rooibos (*Aspalathus linearis*) is a South African fynbos plant with known health-promoting properties, consumed mainly as a herbal tea. The health-promoting properties of rooibos are associated with its phenolic composition. During herbal tea production, the plant material is "fermented", which reduces the phenolic content. This led to development of unfermented (green) rooibos tea with increased phenolic content. Conventionally the phenolic compounds are quantitatively and qualitatively analysed using reversed phase (RP) high performance liquid chromatography (HPLC) with diode array detection (DAD) and mass spectrometry (MS).

This thesis reports the design of a validated quantitative RP-HPLC-DAD method to enable quantification of four eriodictyol-glucopyranoside isomers that have not been previously quantified in rooibos. External authentic reference standards were used to identify and quantify phenolic compounds with the help of MS, which also confirmed peak purity for the quantified compounds. The plant material of 10 rooibos plants were sub-divided before processing prepare green, semi-fermented and fermented products from each. Mixtures of acetonitrile and ethanol with water (0, 20, 40, 60, 80 and 100%) were evaluated for maximal extraction of phenolics from the plant material. Extracts prepared with 40% acetonitrile representing maximal extraction from the plant material, as well as water extracts (food ingredient extracts), were analysed. For the first time aspalathin was quantified in rooibos with its known degradation products, the eriodictyol-glucopyranoside isomers, iso-orientin and orientin. In addition, a phenylpropanoid and eight other phenolic compounds were also quantified.

Complex natural samples such as rooibos contain a range of phenolic compounds, some of which remain unidentified due to challenges in their separation. This led to the development of a comprehensive two dimensional (2D) separation technique to gain in-depth qualitative information on the phenolic composition of rooibos. Normal phase (NP) high performance countercurrent chromatography (HPCCC) was used to develop the first dimension (^{1}D) separation. A gradient elution using an ethyl acetate, *n*-butanol, water solvent system was used to separate the phenolic compounds followed by an extrusion step (60 min analysis time, 48 fractions collected). The second dimension (^{2}D) separation used ultra (U)HPLC to ensure rapid analysis and maximum efficiency. The ^{2}D separation method was developed from the quantitative method with further development aimed at obtaining a high practical peak capacity in a reasonable analysis time. The practical peak capacity was determined as a function of the ^{2}D flow-rate and gradient time, as well as the ^{1}D fraction collection time. The off-line NP-HPCCC×RP-UHPLC method was applied to green and fermented rooibos samples. DAD was used to construct contour plots to elucidate

quantitative and qualitative differences, while MS detection was used for tentative identification of previously unidentified compounds. A total of 39 compounds, 18 of which were not previously identified in rooibos, were identified using MS detection in positive ionisation mode. Most of the newly identified compounds were very polar. The combination of NP-HPCCC with RP-UHPLC separations was characterised by a high degree of orthogonality (~80%), contributing to a high practical peak capacity (3293) and improved separation of especially the novel polar phenolic compounds.

Opsomming

Rooibos (*Aspalathus linearis*) is ‘n Suid-Afrikaanse fynbosplant met gesondheidsvoordele wat merendeels gebruik word as ‘n kruietee. Die gesondheidvoordele van rooibos word geassosieer met sy fenoliese samestelling. Tydens produksie van die tee word die plant materiaal ge-“fermenteer”, wat die fenoliese inhoud verminder. Dit het gelei tot ontwikkeling van ongefermenteerde (groen) rooibostee met hoër fenoliese inhoud. Konvensionele kwalitatiewe en kwantitatiewe analiese van fenoliese verbindings behels omgekeerde fase (RP) hoë-druk vloeistof chromatografie (HPLC) met ultraviolet-fotodiode deteksie (DAD) en massa spektrometrie (MS).

Hierdie tesis beskryf die ontwerp van ‘n gevalideerde RP-HPLC-DAD tegniek om vier eriodictyol-glukopiranosiel isomere vir die eerste keer in rooibos te kan kwantifiseer. Die verbindings is geïdentifiseer en gekwantifiseer deur gebruik te maak van eksterne outentieke standaarde m.b.v. MS, wat ook die suiwerheid van die pieke bevestig het. Plant materiaal van tien rooibos plante is voor prosessering onderverdeel om ‘n groen, semi-gefermenteerde en gefermenteerde produk van elk te berei. Mengsels van asetonitriel en etanol met water (0, 20, 40, 60, 80 en 100%) is ondersoek om maksimale ekstraksie van fenole uit die plant materiaal te verkry. Ekstrakte gemaak met 40% asetonitriel wat maksimale fenoliese ekstraksie uit die plant materiaal verteenwoordig, asook water ekstrakte (voedselbestandeel ekstrakte), is geanaliseer. Aspalatien is vir die eerste keer saam met bekende oksidasieprodukte, naamlik vier eriodictyol-glukopiranosiel isomere, iso-orientin en orientin, in rooibos gekwantifiseer. ‘n Fenielpropanoïed en agt ander fenoliese verbindings is ook gekwantifiseer.

Komplekse natuurlike monsters soos rooibos bevat verskeie fenoliese verbindings, waarvan sommige nog nie geïdentifiseer is nie a.g.v. uitdagings om hulle te skei. Gevolglik is ‘n kwalitatiewe, omvattende, af-lyn, twee-dimensionele (2D) skeidingsmetode ontwikkel om in-diepte kwalitatiewe inligting te verskaf. ‘n Normale fase (NP) hoë werkverrigting vloeistof-vloeistof chromatografie (HPCCC) metode is as eerste dimensie (^{1}D) skeiding ontwikkel. Gradiënt eluering met ‘n oplosmiddelstelsel bewtaande uit etielasetaat, n-butanol en water is gebruik om die fenoliese verbindings te skei gevolg deur verplasing van die stasionêre fase (60 min analisetyd, 48 fraksies opgevang). RP ultra (U)HPLC is gebruik vir die tweede dimensie (^{2}D) skeiding om vinnige analisetyd en effektiewe skeiding te verseker. Die ^{2}D metode is ontwikkel vanaf die kwantitatiewe metode met verdere ontwikkeling met die doel om ‘n hoë praktiese piekkapasiteit in ‘n redelike analisetyd te behaal. Die praktiese piekkapasiteit is bepaal as ‘n funksie van die ^{2}D vloeispoed en gradiënttyd sowel as die ^{1}D fraksie opvangtyd. Die af-lyn NP-HPCCC×RP-UHPLC metode is toegepas op groen en gefermenteerde rooibos monsters. DAD is gebruik om kontoerplotte te genereer waarmee

kwantitatiewe en kwalitatiewe verskille bepaal kon word, terwyl MS deteksie gebruik is vir tentitatiewe identifikasie van voorheen onbekende verbindings. Identifikasie van ‘n totaal van 39 verbindings, waarvan 18 nog nie vantevore in rooibos geïdentifiseer is nie, is moontlik deur gebruik van MS analiese met positiewe ionisasie. Meeste van die nuut geïdentifiseerde verbindings was baie polêr. Die kombinasie van NP-HPCCC en RP-UHPLC skeidings is gekenmerk deur ‘n hoë graad van ortogonaliteit (~80%), wat bygedra het tot ‘n hoë praktiese piekkapasiteit (3293) en verbeterde skeiding van veral nuwe polêre fenoliese verbindings.

ACKNOWLEDGMENTS

I would like to thank and give my gratitude to the following people and institutions:

My supervisor, Dr. Dalene de Beer and my Co-supervisors, Prof. André de Villiers and Dr. Paul Williams. Thank you for the guidance, patience and advice to help me complete this thesis. It has been my privilege to work under such an excellent group of researchers, each with outstanding character.

Prof. Elizabeth Joubert, (Post-Harvest and Wine Technology Division, ARC Infruitec-Nietvoorbij) for providing valuable assistance and inspiring me every day with your dedication to research the “never give up” example you set for us all.

Dr. Christiaan (Christie) Malherbe (Post-Harvest and Wine Technology Division, ARC Infruitec-Nietvoorbij), for providing practical assistance, teaching me how to do basic repairs for machinery, always having encouraging words and a sense of humour.

Dr. Maria Stander (Central Analytical Facility, Stellenbosch University), for assisting with the LC-MS analyses.

Marieta van der Rijst (Biometry Unit, ARC Infruitec-Nietvoorbij), for the statistical data analysis.

Carin De Wet (Post-Harvest and Wine Technology Division, ARC Infruitec-Nietvoorbij) for providing admin assistance and keeping everyone’s head above water.

George Dico (Post-Harvest and Wine Technology Division, ARC Infruitec-Nietvoorbij) for sample preparation and general assistance in the work area.

Guy Emerton, for providing a Macro to simultaneously extract multiple data files from Chemstation as csv files.

Willie Pretorius and Magriet Muller, for assisting me in writing MATLAB scripts.

I am indebted to the National Research Foundation (NRF) Competitive Programme for Rated Researcher (grant nr 93490) to Dr Dalene de Beer. Opinions expressed and

conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

I am extremely grateful for the Agricultural Research Council's Professional Development Program (PDP) funding, which allowed me to study and complete my MSc in food science.

I would like to thank God, for guiding and protecting me every day of my life.

My friends and family for the support and pressuring me at the right moments and listening to me when I ramble on about the work and occasionally laughing at my dry sense of humour.

NOTES

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion, recommendations and conclusions. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

Table of contents

Chapter 1

General introduction

Rooibos (*Aspalathus linearis*), a fynbos species indigenous to South Africa, is cultivated in the Clanwilliam region and can be naturally found in the Cederberg area (Joubert *et al.*, 2008a). Rooibos is processed to produce a herbal tea known as rooibos tea. Rooibos extracts are also added to many other products, such as cosmetics, yoghurt, bread and iced tea (Joubert & De Beer, 2011).

Beneficial properties of rooibos include anti-mutagenic, anti-oxidant, anti-diabetic and anti-microbial activities as reviewed in Joubert *et al.*, (2008a) and Muller *et al.*, (2016), which are being studied to further our understanding of these attributes. When added to baby formula or administered to infants, rooibos tea has been shown to reduce the symptoms of colic, stimulate appetite and reduce allergenic reactions (Joubert *et al.*, 2008a).

Rooibos fermentation is an oxidative process presumably initiated by plant enzymes in the presence of oxygen and water (Bramati *et al.*, 2002) and not by micro-organisms, usually associated with fermentation (Gouws *et al.*, 2014). Rooibos fermentation reduces the anti-oxidant capacity of the extracts, due to the lowered polyphenolic content (Joubert *et al.*, 2008b). Due to this loss in polyphenols, unfermented rooibos is generally regarded as healthier. A major polyphenolic compound present in rooibos, aspalathin, has been linked with much of the beneficial properties of rooibos. Aspalathin is a dihydrochalcone that has to date only been found in rooibos (Koeppen & Roux, 1965). This compound is extremely susceptible to oxidation, and during fermentation almost 98% of the initial aspalathin content is lost due to oxidation (Schulz *et al.*, 2003). Oxidation products formed include (*R*)/(*S*)-eriodictyol-6-*C*-glucopyranoside, (*R*)/(*S*)-eriodictyol-8-*C*-glucopyranoside, orientin, iso-orientin and red/brown polymers (Krafczyk *et al.*, 2009; Heinrich *et al.*, 2012).

Marais *et al.*, (2000) found (*R*)- and (*S*)-eriodictyol-6-*C*-glucopyranoside isomers in processed rooibos plant material, and suggested that these flavanones are formed via oxidative cyclisation of aspalathin. Krafczyk *et al.*, (2009) showed that (*R*)- and (*S*)-eriodictyol-8-*C*-glucopyranoside isomers were present in isolated aspalathin solutions which were heated to 37 °C for 24 h in a shaker incubator. These studies provided strong evidence that the eriodictyol-glucopyranoside isomers found in rooibos extracts were formed as a degradation product of aspalathin during fermentation. The lack of a suitable analytical method to simultaneously separate and quantify aspalathin, the eriodictyol-glucopyranoside isomers, iso-orientin and orientin leaves a gap in research. Most studies regarding quantification of rooibos phenolics focus on major phenolic compounds, and not on minor degradation products formed during the fermentation process. An analytical high performance liquid chromatography (HPLC) diode array detection (DAD) method described by De Beer *et al.*, (2015) is currently used by our group to rapidly (16 min) quantify aspalathin, nothofagin, iso-orientin and orientin. Another HPLC-DAD method previously reported for quantification of 15 major rooibos phenolics by Beelders *et al.*, (2012b) could not

separate and quantify the eriodictyol-glucopyranoside isomers. Bramati *et al.*, (2002) developed a quantitative HPLC-DAD method for the quantification of 10 major phenolic compounds present in rooibos plant material, but the method was also not suitable for quantification of the eriodictyol-glucopyranoside isomers. The development and application of a suitable HPLC method to accurately quantify aspalathin, the eriodictyol-glucopyranoside isomers, iso-orientin and orientin is essential to further increase our understanding of aspalathin degradation during the fermentation process.

In addition to the effect of processing on the phenolic composition of rooibos, genetic differences (rooibos reproduce from seedlings) and varying geographic/ecological environments might result in different quantities of phenolic compounds, including eriodictyol-glucopyranosides, produced by the plant (Joubert & De Beer, 2011). These differences must be quantified using reproducible and accurate analytical techniques to enable future research on the effects of eriodictyol-glucopyranoside isomers in rooibos plant material on rooibos flavour and/or bioactivity.

Complex samples such as rooibos, which contain large numbers of polyphenolic compounds, places extreme demands on the performance of one-dimensional (1D) HPLC (Kalili & De Villiers, 2013). The finite chromatographic performance of 1D-HPLC, coupled to limited availability of commercial standards and challenges encountered in the identification of low-level unknown compounds is responsible for the fact that quantitative data for rooibos are limited to a relatively small number of major phenolic compounds. Especially information on the most polar compounds, which elute early in conventional reversed phase (RP) HPLC methods, is still lacking.

To overcome the performance limitations of 1D-HPLC, two-dimensional (2D) LC may be used, where the two separations utilise orthogonal separation mechanisms (Pourhaghighi *et al.*, 2011). To date, only a single report on the 2D-LC analysis of rooibos has been reported to obtain comprehensive qualitative information regarding rooibos phenolic compounds (Beelders *et al.*, 2012a).

Countercurrent chromatography (CCC), which is a separation mode based on liquid mobile and stationary phases, is an ideal first dimension (^{1}D) preparative separation technique in combination with HPLC (Berthod *et al.*, 2009). As mobile and stationary phases, collectively referred to as the solvent system, are both liquids in CCC, only liquid-liquid partitioning is responsible for separation and theoretically 100% of the sample analytes can be recovered after separation. The only prerequisite for a suitable solvent system in CCC is that there should always be more than one liquid phase. A near limitless choice of solvent systems is therefore available, thereby providing a wide range of options to affect selectivity ranges for sample analytes (Friesen & Pauli, 2005). Countercurrent chromatography has previously been used to successfully separate phenolic compounds as a preparative method

(Chen *et al.*, 2015; De Beer *et al.*, 2015; Liu *et al.*, 2015). Two-dimensional (2D) analysis combining CCC in the ^{1}D in conjunction with RP-HPLC in the second dimension (^{2}D) should provide a highly orthogonal combination to efficiently perform comprehensive qualitative analyses of rooibos phenolic compounds.

In light of the above, the first research aim of this work was to development a quantitative RP-HPLC-DAD method suitable for quantification of aspalathin, the four eriodictyol-glucopyranoside isomers, iso-orientin and orientin, as well as many as possible of the major rooibos polyphenolic compounds. This method will be applied to the quantitative analysis of ten green, semi-fermented and fermented rooibos water extracts originating from the same randomly selected rooibos bush. As part of this work, the extraction solvent will be optimised for the extraction of the maximum levels of phenolic compounds, in order to obtain their content in the plant material.

The second research aim was to design a qualitative (off-line) comprehensive 2D CCC×LC method for the detailed qualitative analysis of rooibos phenolics. This method will be applied to a green and fermented extract in conjunction with DAD to construct contour plots. In addition, mass spectrometric (MS) and tandem MS (MS/MS) detection will be used to tentatively identify peaks.

References

Beelders, T., Kalili, K. M., Joubert, E., De Beer, D. & De Villiers, A. (2012a). Comprehensive two-dimensional liquid chromatographic analysis of rooibos (*Aspalathus linearis*) phenolics. *Journal of Separation Science,* **35**, 1808-1820.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012b). Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation. *Journal of Chromatography A,* **1219**, 128-139.

Berthod, A., Maryutina, T., Spivakov, B., Shpigun, O. & Sutherland, I. A. (2009). Countercurrent chromatography in analytical chemistry (IUPAC technical report). *Pure and Applied Chemistry,* **81**, 355-387.

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P. & Pietta, P. (2002). Quantitative characterization of flavonoid compounds in rooibos tea (*Aspalathus linearis*) by LC-UV/DAD. *Journal of Agricultural and Food Chemistry,* **50**, 5513-5519.

Chen, W. B., Li, S. Q., Chen, L. J., Fang, M. J., Chen, Q. C., Wu, Z., Wu, Y. L. & Qiu, Y. K. (2015). Online polar two phase countercurrent chromatography x high performance liquid chromatography for preparative isolation of polar polyphenols from tea extract in a single step. *Journal of Chromatography B,* **997**, 179-186.

De Beer, D., Malherbe, C. J., Beelders, T., Willenburg, E. L., Brand, D. J. & Joubert, E. (2015). Isolation of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) using high-performance countercurrent chromatography: Sample loading and compound stability considerations. *Journal of Chromatography A,* **1381**, 29-36.

Friesen, B. & Pauli, G. F. (2005). G.U.E.S.S.—A generally useful estimate of solvent systems for CCC. *Journal of Liquid Chromatography & Related Technologies,* **28**, 2777-2806.

Gouws, P., Hartel, T. & Van Wyk, R. (2014). The influence of processing on the microbial risk associated with rooibos (*Aspalathus linearis*) tea. *Journal of the Science of Food and Agriculture,* **94**, 3069-3078.

Heinrich, T., Willenberg, I. & Glomb, M. A. (2012). Chemistry of color formation during rooibos fermentation. *Journal of Agricultural and Food Chemistry,* **60**, 5221-5228.

Joubert, E. & De Beer, D. (2011). Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany,* **77**, 869-886.

Joubert, E., Gelderblom, W. C. A., Louw, A. & De Beer, D. (2008a). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Journal of Ethnopharmacology,* **119**, 376-412.

Joubert, E., Richards, E. S., Van der Merwe, J. D., De Beer, D., Manley, M. & Gelderblom, W. C. A. (2008b). Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). *Journal of Agricultural and Food Chemistry,* **56**, 954-963.

Kalili, K. M. & De Villiers, A. (2013). Systematic optimisation and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography x reversed phase liquid chromatographic analysis of procyanidins, part I: Theoretical considerations. *Journal of Chromatography A,* **1289**, 58-68.

Koeppen, B. H. & Roux, D. G. (1965). Aspalathin: A novel *C*-glycosylflavanoid from *Aspalathus linearis*. *Tetrahedron Letters,* **39**, 3497-3503.

Krafczyk, N., Heinrich, T., Porzel, A. & Glomb, M. A. (2009). Oxidation of the dihydrochalcone aspalathin leads to dimerization. *Journal of Agricultural and Food Chemistry,* **57**, 6838-6843.

Liu, Q., Zeng, H., Jiang, S., Zhang, L., Yang, F., Chen, X. & Yang, H. (2015). Separation of polyphenols from leaves of *Malus hupehensis* (Pamp.) Rehder by off-line two-dimensional high speed counter-current chromatography combined with recycling elution mode. *Food Chemistry,* **186**, 139-145.

Marais, C., Janse van Rensburg, W., Ferreira, D. & Steenkamp, J. A. (2000). (*S*)- and (*R*)-Eriodictyol-6-*C*-β-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*). *Phytochemistry,* **55**, 43-49.

Muller, C. J., Malherbe, C. J., Chellan, N., Yagasaki, K., Miura, Y. & Joubert, E. (2016). Potential of rooibos, its major *C*-glucosyl flavonoids and Z-2-(beta-D-glucopyranoloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. *Critical Reviews in Food Science and Nutrition,* DOI: 10.1080/10408398.2016.1157568.

Pourhaghighi, M. R., Karzand, M. & Girault, H. H. (2011). Orthogonality of two-dimensional separations based on conditional entropy. *Analytical Chemistry,* **83**, 7676-7681.

Schulz, H., Joubert, E. & Schütze, W. (2003). Quantification of quality parameters for reliable evaluation of green rooibos (*Aspalathus linearis)*. *European Food Research and Technology,* **216**, 539-543.

Chapter 2

Literature review

The literature review chapter will start with a brief overview of rooibos (*Aspalathus linearis*) focussing on the polyphenols present in rooibos. The various health benefits associated with rooibos and rooibos polyphenols will be briefly reviewed. The stability of rooibos polyphenols, especially during processing to produce the traditional fermented tea, will also be discussed. Since the analysis of rooibos polyphenols is important, liquid chromatographic techniques, namely high performance liquid chromatography (HPLC), countercurrent chromatography (CCC) and two-dimensional (2D) chromatography, as well as detection using UV/Vis and mass spectrometry (MS), will be covered in detail.

1. Rooibos Background, Various Uses and Benefits

1.1. Introduction

One of South Africa's well-known fynbos species, rooibos (*Aspalathus linearis*), is a caffeine free, low tannin herbal tea originating from South Africa and is mainly cultivated around the Clanwilliam region, but also in Nieuwoudtville in the Northern Cape. More than 270 other *Aspalathus* species exist, but do not have any commercial value and might be threatened by extinction due to selective breeding and expanding of rooibos plantations in their natural habitat. Rooibos ecotypes have different morphology ranging from tree-types to shrub-types, each appearing in different geographical areas. The tree types usually appear near water sources and the shrub type at drier rocky areas (Malgas *et al.*, 2010). Today the only rooibos type with commercial value is the Rockland type or Red type rooibos. This Rockland rooibos can be further classified as the Cederberg type (wild growing) or Nortier type (cultivated). The cultivated type should have a characteristic quality: when bruised, the leaves will turn red/brown. Rooibos plants are cultivated from seedlings which introduce genetic variation, thereby resulting in varying polyphenolic content of different plants (as reviewed by Joubert & De Beer, 2011).

1.2. Rooibos Processing

Due to historical reasons rooibos processing is referred to as “fermentation”, although microbial organisms are not primarily responsible for the organoleptic and chemical changes occurring during this process. Per definition, oxidation is when reactive oxygen species attack susceptible molecules, while fermentation is a biological process whereby yeast or bacteria produce alcohol and lactic acid from sugars and carbohydrates in an oxygenated or deoxygenated environment. The change in colour when rooibos plant material is bruised is due to oxidation reactions taking place via enzymatic and chemical pathways at 38-42 °C (Joubert *et al.*, 2008).

Rooibos is harvested at plantations by cutting the plant just above the topping, followed by cutting the leaves and stems into small pieces. The cut rooibos is then placed in a fermentation heap outside, allowing exposure to open air. The rooibos is then wetted, bruised and rolled out to further disrupt the cell walls and allow enzymes to be released from cells. Periodically the fermentation heaps are turned over to aerate the fermenting rooibos. These processes speed up the oxidation reactions and lower the overall fermentation time. The green rooibos rapidly undergoes a colour change to red/brown during the fermentation process (Joubert & De Beer, 2014). **Figure 2.1** presents a concise overview of the steps involved in rooibos processing.

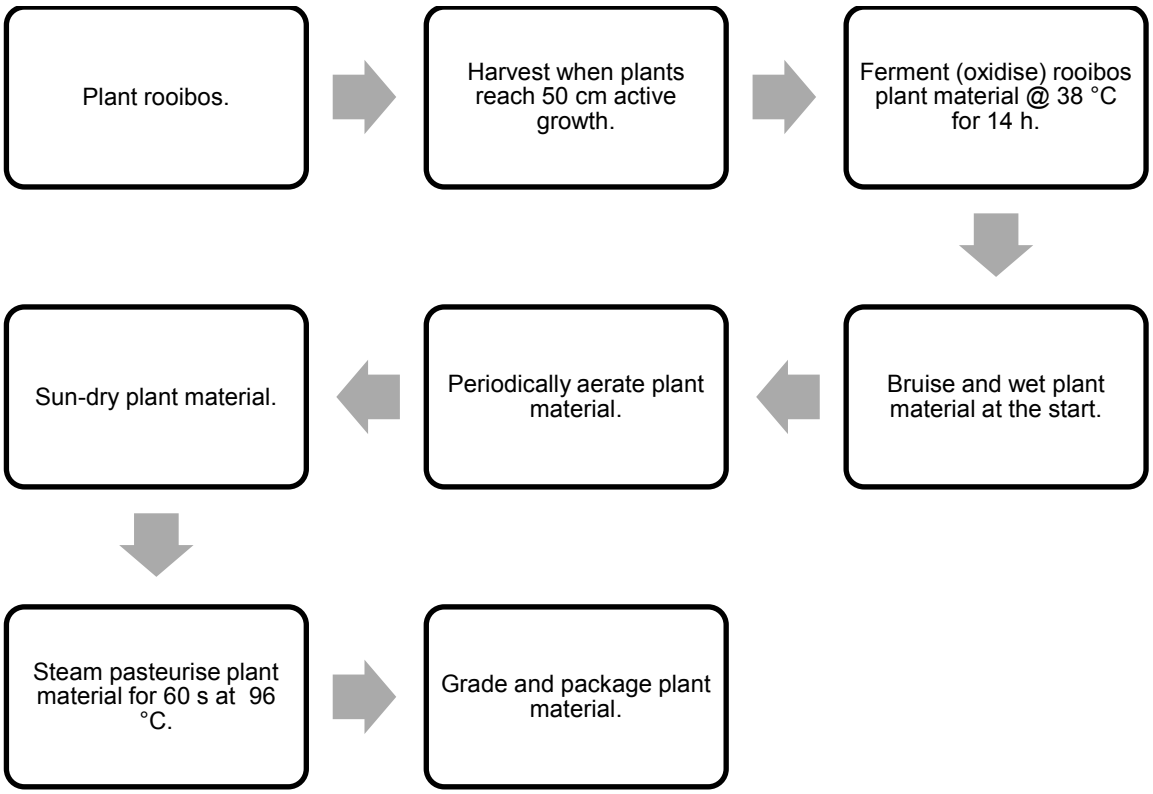


Figure 2.1 A schematic overview of the processing of rooibos plant material for fermented rooibos products (Koch *et al.*, 2013; Joubert & De Beer, 2014).

During fermentation, aspalathin, a major polyphenol present in rooibos, is oxidised and after 3.5 h, only 32% of the initial aspalathin content remains (Joubert, 1996). Fermentation of rooibos should not be continued longer than 14 h as the polyphenolic content will be drastically reduced. After fermentation the rooibos is spread out to allow rapid sun-drying. Poor aeration of rooibos during fermentation produces low quality rooibos (Joubert *et al.*, 2008; Joubert & De Beer, 2014).

Green rooibos products are produced by excluding the fermentation step and minimising oxidation. Green rooibos products were first produced in 2001 as a demand was present for a rooibos product with a higher anti-oxidant capacity. Green rooibos is produced by cutting the shoots and rapidly air-drying the plant material to stop the oxidation reactions during fermentation. If done incorrectly, the content of aspalathin and other polyphenols decreases over time and slow browning occurs (Joubert *et al.*, 2008).

1.3. Steam Pasteurisation

Like any plant product, especially since processing is performed in the open air, processed rooibos plant material contains a high microbial load. One of the microbial organisms present on rooibos plant material is *Salmonella typhimurium*, which is a human pathogen that is unable to tolerate high temperatures, thereby making steam pasteurisation a viable strategy for the reduction of the microbial load in rooibos products (Gouws *et al.*, 2014).

In the 1980's a steam pasteurisation technique was developed for rooibos to avoid *Salmonella* contamination, where the rooibos was subjected to steam at 96 °C for 60 s in order to reduce the microbial load to acceptable levels (Joubert & De Beer, 2011; Koch *et al.*, 2013). As rooibos polyphenols are very susceptible to heat and oxidation, antimicrobial treatment such as pasteurisation can affect the phenolic composition and also the sensory properties. Steam pasteurisation was shown to change the organoleptic profile of the rooibos by reducing most flavours by a small amount and producing a new medicinal flavour. When investigating the effects of steam pasteurisation on polyphenols it was found to only significantly reduce the aspalathin content. As aspalathin is converted to iso-orientin and orientin, any losses of iso-orientin and orientin will be replenished by the oxidation of aspalathin. Colour and organoleptic changes have been noted to take place during steam pasteurisation. Koch *et al.*, (2013) found that the aroma associated with hay also tends to increase during steam pasteurisation; except for grade A rooibos product, all other flavour and aromatic traits seem to decrease.

Currently studies are being performed to investigate the use of lactic acid bacteria as a biocontrol agent during the fermentation process to help control pathogenic microbial numbers as *Salmonella* has been detected in rooibos previously after pasteurisation (Gouws *et al.*, 2014).

1.4. Quality Control of Rooibos

In the past producers and manufacturers of rooibos products had no official reference to work from when evaluating their products based on sensory aspects. As a result, companies could make various claims about their rooibos product's sensory related quality with no official guideline in place. The characteristic rooibos flavour was not clearly defined (Koch *et al.*, 2012). To remedy this situation, Koch *et al.*, (2012) created a sensory wheel to define the general "rooibos flavour". The sensory wheel encompasses mouthfeel and sensory attributes of rooibos tea products and includes both negative and positive sensory descriptors.

Prior to this study and still in use by industry to grade processed rooibos is a classification system where the rooibos batches are evaluated by expert graders depending on the appearance of the dry and infused rooibos leaves and the colour and flavour of rooibos infusions. Grade A is the highest grade and usually associated with the highest polyphenolic content, and grade D is associated with the lowest polyphenolic content. Grade D is usually sold for extract purposes (Joubert *et al.*, 2012).

As a result of the varying rooibos polyphenolic content between product batches, two of the predominant producers in South African of rooibos extracts use the total anti-oxidant capacity (TAC) and total polyphenol content (TPC) assay as a prospective quality control measure for their extracts (Joubert & De Beer, 2011).

1.5. Beneficial Uses of Rooibos

Rooibos extracts are rich in polyphenolic compounds and it is well known that polyphenols are excellent anti-oxidants (Almajano *et al.*, 2008). The same can be said about the polyphenols present in rooibos. Polyphenols in rooibos have been reported to present anti-oxidant properties in a range of assays (Von Gadow *et al.*, 1997; Joubert *et al.*, 2005; Kawano *et al.*, 2009; Marnewick *et al.*, 2009; Snijman *et al.*, 2009; Marnewick *et al.*, 2011). These anti-oxidant properties are believed to lead to various health benefits such as anti-diabetic, anti-carcinogenic and anti-inflammatory effects (Joubert & De Beer, 2011). Although most of these health benefits were only observed *in vitro, in vivo* experiments are becoming more prominent and showing more significant results as technological advances are achieved (Marnewick *et al.*, 2005; Dludla *et al.*, 2014; Hoffman *et al.*, 2014; Hübsch *et al.*, 2014; Jones *et al.*, 2015; Ku *et al.*, 2015; Kwak *et al.*, 2015).

Various studies have been performed in the past few years on the anti-diabetic effect of rooibos polyphenols (Kawano *et al.*, 2009; Mazibuko *et al.*, 2013; Dludla *et al.*, 2014; Ku *et al.*, 2015). Diabetes mellitus is a metabolic disorder that is becoming a global epidemic which affects the way carbohydrates, proteins and lipids are absorbed in the human body. These symptoms are caused by the body not being able to produce enough insulin or being resistant to the insulin produced. Studies performed by multiple researchers found positive

results when testing the effect of rooibos extracts on glucose absorption (Kawano *et al.*, 2009; Muller *et al.*, 2012; Mazibuko *et al.*, 2013). The increased uptake of glucose in the blood is because the polyphenols, especially aspalathin, can protect the pancreas from oxidative stress, thereby allowing the organ to secrete more insulin (Kawano *et al.*, 2009). Aspalathin was found to increase glucose uptake in muscle cells with increased activity when used in combination with rutin (Kamakura *et al.*, 2015). The effect of aspalathin on glucose uptake is dose dependant in the absence of insulin (Kawano *et al.*, 2009).

Researchers are also investigating the use of rooibos in order to prevent or reduce the growth of cancer. Unfermented rooibos was found to be more effective than fermented rooibos for the inhibition and/or reduction of tumorous skin growths in a mouse model (Marnewick *et al.*, 2005). This might be because aspalathin is an excellent anti-oxidant and high levels of this polyphenol are present in the green rooibos samples. Rooibos extracts were also fed to rats in order to determine if the anti-mutagenic properties of the polyphenols can reduce the occurrence of cancer in rats. In a subsequent study by Marnewick *et al.*, (2009), it was found that fermented and green rooibos showed reduction in oxidative stresses present in cells, but the rooibos acted as a pro-oxidant during polyphenol/FB1/iron interactions. They concluded that fermented rooibos tea resulted in a lower protection against oxidative stresses due to the lower polyphenol content (Marnewick *et al.*, 2009).

All the *in vitro* properties of rooibos polyphenols appear promising, but in order for rooibos to be of use to humans it must be bioavailable and absorbed into the body. Villaño *et al.*, (2010) studied rooibos tea and the effect it has on the anti-oxidant content in human blood plasma when consumed. It was concluded that rooibos tea consumption increases the anti-oxidant content of blood plasma. This was tested using the total radical trapping anti-oxidant potential (TRAP) assay. The mechanism for this assay is to determine how much oxygen is consumed during controlled lipid oxidation due to heat degradation. The accuracy of the assay would be lowered if plasma proteins are present, as polyphenols bind to these proteins preventing detection (Ghiselli *et al.*, 1994). A similar study was performed by Breiter *et al.*, (2011) on 12 males whose major source of anti-oxidants was rooibos tea - they were instructed to consume other foods low in anti-oxidants. Instead of using the TRAP assay, the oxygen radical absorbance capacity (ORAC) assay was used in the latter study. Rooibos polyphenols did not have a significant effect on the anti-oxidant levels of test subjects and were poorly bioavailable. It has been reported that the ORAC assay is not a perfect *in vivo* test, as polyphenols bound to proteins are not detected with this assay. Currently there is not a single total anti-oxidant capacity (TAC) assay available that is perfect for *in vivo* studies.

Only a small fraction of the polyphenols in the human diet will be absorbed while still retaining their natural form, as polyphenols are metabolised by the body (Crozier *et al.*, 2009). With low bioavailability, other non-*in vivo* uses for rooibos polyphenols have also

been researched, such as using their anti-microbial and anti-oxidant activity to protect food products against spoilage (Hoffman *et al.*, 2014; Jones *et al.*, 2015).

Wastage of food is a global problem as large populations lack sufficient nourishment. Much of the food processed in the world goes to waste due to microbial spoilage. Researchers have been pursuing studies on the anti-microbial effects of rooibos (Oh *et al.*, 2013; Hübsch *et al.*, 2014). During the process of studying the effect of rooibos on bacteria, Almajano *et al.*, (2008) stated that gram negative bacteria were more resistant to rooibos than gram positive bacteria when used in conjunction with antibiotics. This might be due to the difference in cell wall structures or the specific bacterial strains. A different study done by Coetzee *et al.*, (2008) to test the anti-microbial effects of rooibos on *Botrytis cinerea*, found that rooibos inhibits spore germination, but increased the growth of the vegetative cells. The latter effect was ascribed to the presence of minerals and nutrients in the rooibos extract that might promote growth. It was also found that rooibos had a bacteriostatic effect on *E. coli* during this study, which is contradictory to the results found by Almajano *et al.*, (2008). This can be because different strains of *E. coli* might have been used in the two studies.

Lipid peroxidation in the meat industry is a serious problem, because lipid peroxidation sets in motion a chain reaction which escalates the oxidation rate. Rooibos polyphenols that act as natural anti-oxidants can in theory delay the onset of lipid peroxidation. The effect of rooibos on protection of meat products from lipid peroxidation was first studied by (Cullere *et al.*, 2013). They found that unfermented and fermented rooibos could increase the shelf-life of ostrich meat patties and retard lipid peroxidation, respectively. Droëwors is such a product that is susceptible to lipid peroxidation and also protein oxidation. Droëwors is traditionally made from meat and animal fat, followed by a drying period (Hoffman *et al.*, 2014; Jones *et al.*, 2015). At low water activities (a_w <0.2) lipid peroxidation will be accelerated, which can explain why droëwors is a prime candidate for lipid peroxidation (**Figure 2.2**). Hoffman *et al.*, (2014) showed that rooibos extracts did not reduce lipid peroxidation in droëwors made from ostrich meat and pork back fat, but rather acted as a pro-oxidant, especially at higher concentrations. Another study by Jones *et al.*, (2015) using Springbok and Blesbok meat added with beef to increase the fat content of droëwors found the opposite to be true when rooibos was incorporated: in this study a higher concentration of rooibos extracts led to a higher degree of protection against oxidation. Both of these studies did not investigate the phenolic composition of the rooibos extracts that were used.

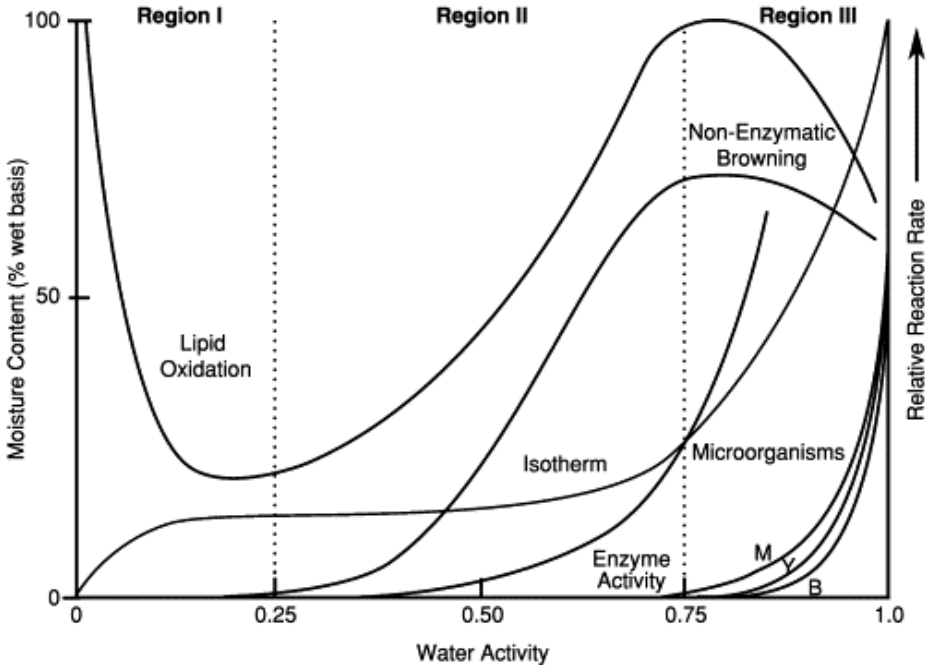


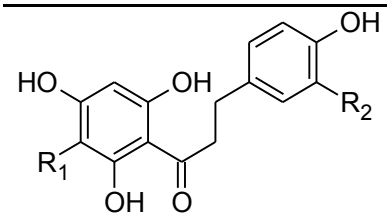
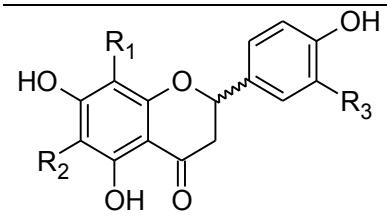
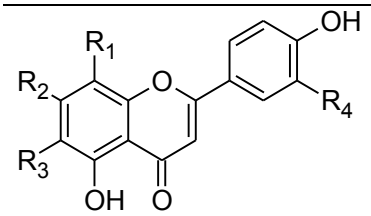
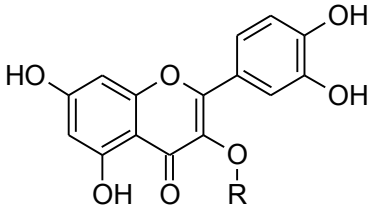
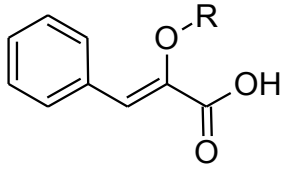
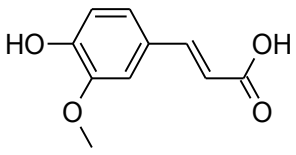
Figure 2.2 Illustration of water activity to moisture content (% wet basis) on how lipid oxidation will accelerate when an a_w <0.2 is obtained (Labuza & Dugan, 1971).

1.6. Rooibos Phenolic Composition

Polyphenols are stress-induced secondary metabolites used in plant defence. Plants produce these molecules in adverse conditions, implying that the more hostile the environmental effects are for a plant, the more polyphenols will be present in the plant cells. Polyphenols protect the plant against oxidation and can act as natural pesticides (Brandt & Mølgaard, 2001; Kazimierczak *et al.*, 2013).

Rooibos has a variety of polyphenols with most of them classified in the following four classes: dihydrochalcones, flavanones, flavones and flavonols. Phenylpropanoid and hydroxycinnamic acid compounds have also been identified in rooibos samples (Joubert & De Beer, 2011). **Table 2.1** shows names and structures of the major phenolic compounds found in rooibos.

Table 2.1 Major phenolic compounds found in rooibos (Beelders *et al.*, 2012a; Joubert & De Beer, 2014)

Structure	Compound type, name	Substituents
	**Dihydrochalcones**	
	Aspalathin	R_1 = β-D-glucosyl, R_2 = OH
	Nothofagin	R_1 = β-D-glucosyl, R_2 = H
	**Flavanones**	
	Hemiphlorin	R_1 = β-D-glucosyl, R_2 =R_3=H
	(*R*)/(*S*)-eriodictyol-8-*C*-glucoside:	R_1 = β-D-glucosyl, R_2 = H, R_3 = OH
	(*R*)/(*S*)-eriodictyol-6-*C*-glucoside:	R_1 = H, R_2 = β-D-glucosyl, R_3 = OH
	**Flavones**	
	Orientin	R_1 = β-D-glucosyl, R_2 = R_4 =OH, R_3 = H
	Iso-orientin	R_1 = H, R_2 = R_4 = OH, R_3 = β-D-glucosyl
	Vitexin	R_1= β-D-glucosyl, R_2 = OH, R_3 = R_4 = H
	Isovitexin	R_1 = R_4 =H, R_2 = OH, R_3 = β-D-glucosyl
	Luteolin-7-*O*-glucoside	R_1 = R_3 = H, R_2 = *O*-β-D-glucosyl, R_4 = OH
	**Flavonols**	
	Isoquercitrin	R = β-D-glucosyl
	Hyperoside	R = β-D-galactosyl
	Rutin	R = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl
	Quercetin-3-*O*-robinobioside	R = α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl
	**Phenylpropanoid**	
	Enolic phenylpyruvic acid-2-*O*-glucoside	R = β-glucosyl
	**Hydroxycinnamic acid**	
	Ferulic acid	As seen in structure

Flavanones, flavones, dihydrochalcones and flavonols each belong to a collective group called flavonoids. This means that the four subgroups will have some overlapping properties, with some properties being more prominent in certain groups. An example of this is flavonols containing a 3-OH group, which will be planar and have a higher radical scavenging ability as electron delocalisation and conjugation are facilitated. Flavanones and flavones that lack the 3-OH moiety are slightly twisted, and therefore conjugation is restricted and the radical scavenging ability of the compound reduced (Heim *et al.*, 2002). The majority of the polyphenols in rooibos have *C*-glycosidic bonds, with the flavonols, PPAG and luteolin-7-*O*-glucoside having *O*-glycosidic bonds (Beelders *et al.*, 2012b).

Aspalathin is unique to rooibos and nothofagin has only been found in heartwood of *Nothofagus fusca* and the bark of a Chinese medicinal plant *Schoepfia chinensis (Koeppen & Roux, 1965; Hillis & Inoue, 1967).* In general these two dihydrochalcones are known for their anti-oxidant properties *in vitro*, with aspalathin having more prominent anti-oxidant properties. However, the anti-oxidant properties of these compounds *in vivo* is under investigation, since aspalathin and nothofagin have low bioavailability (Van der Merwe *et al.*, 2010). Other dihydrochalcones can almost exclusively be found in apples and apple products such as ciders (Crozier *et al.*, 2009).

Flavonols are the most diverse class flavonoids and can be found in the entire plant kingdom except for algae and fungi (Crozier *et al.*, 2009). In rooibos the quercetin-glycosides can either have monosaccharide or disaccharide side chains attached to the aglycone.

Flavanones are mostly found in citrus fruit and can have various sensory properties. For example, flavanone-rutinosides are tasteless, whereas flavanone-neohesperidosides have a bitter taste (Crozier *et al.*, 2009). Flavanones occurring in rooibos all have *C*-glycosidic bonds.

Flavones are not as widely available in nature as flavanones, but can be found in some herbs, celery, parsley and in some *Citrus* species (Crozier *et al.*, 2009). All the flavones in rooibos are either luteolin or apigenin derivatives. Most of the flavones in rooibos have *C*-glycosidic bonds, except for luteolin-7-*O*-glucoside (Beelders *et al.*, 2012b). Luteolin-di-*C*-glucoside was detected in rooibos extracts and identified as carlinoside (Iswaldi *et al.*, 2011; Beelders *et al.*, 2012b). Carlinoside promotes the elimination of bilirubin from the liver by contributing to the conversion of insoluble bilirubin to its soluble form through glucuronidation (Kundu *et al.*, 2011).

Phenolic acids are non-flavonoids that are present in rooibos plant material. Examples of these compounds include *p*-hydroxybenzoic, protocatechuic, vanillic, caffeic, *p*-coumaric and ferulic acids (Joubert, 1996). Ferulic acid is a major hydroxycinnamic acid found in rooibos plant material. Phenolic acids are poorer radical scavengers than aspalathin (Joubert & De Beer, 2014). Ferulic acid occurs in plant cells and can be covalently linked to

polysaccharides in plant cell walls, thereby playing a role in lignin formation (Kroon & Williamson, 1999).

Rooibos plant material also contains a phenylpropanoid compound namely Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG), which has been postulated to contribute to the taste and mouthfeel of rooibos (Joubert *et al.*, 2013). PPAG is highly photolytically sensitive and when exposed to light will readily degrade (Marais *et al.*, 1996).

During rooibos fermentation the polyphenols undergo oxidation. One of the major polyphenols, aspalathin undergoes oxidation at a more rapid pace than the other compounds, and is degraded into other polyphenolic by-products, such as dibenzofurans or red brown polymers (Krafczyk *et al.*, 2009; Heinrich *et al.*, 2012). The dibenzofurans will break down to high molecular weight tannin-like structures after 60 h, which also contribute to the red-brown colour (Heinrich *et al.*, 2012). Currently research findings suggests that aspalathin is the main contributor to the change in colour, but degradation products of other compounds also cause a red-brown colour, although their contribution to the overall colour change is minimal (Krafczyk *et al.*, 2009).

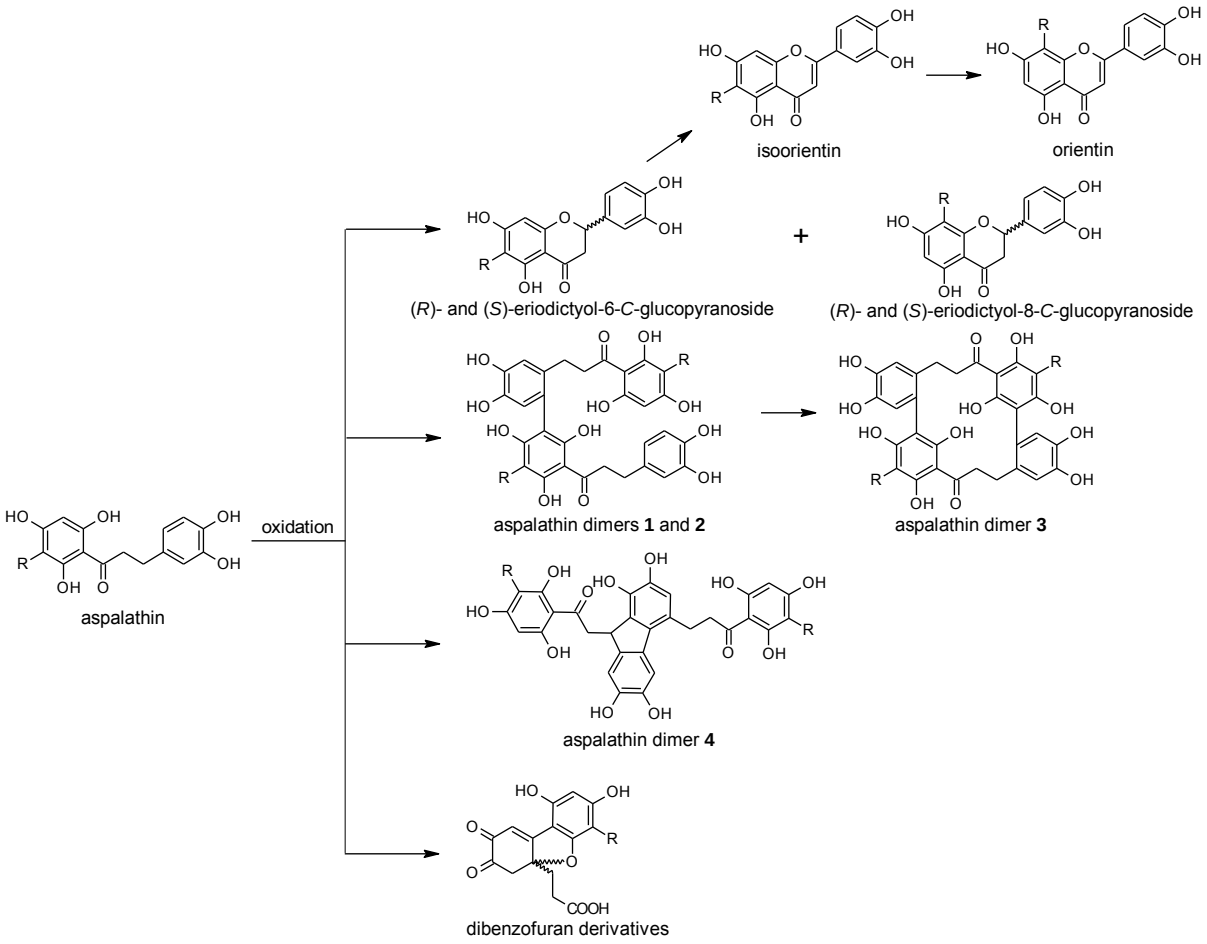


Figure 2.3 Scheme showing aspalathin and its oxidation products formed during fermentation. R = β-D-glucopyranosyl (Joubert & De Beer, 2014).

As summarised in **Figure 2.3,** aspalathin is converted to either (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside, (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside, (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside or (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside. (*R*)- and (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside are then further degraded into iso-orientin, which forms orientin (Krafczyk & Glomb, 2008).

2. High Performance Liquid Chromatography (HPLC)

2.1. Introduction

Chromatography is used to separate compounds in mixtures based on their different properties. Separation science is an important component in any modern laboratory as it allows quantification of separated compounds and identification of unknown compounds.

Chromatography is a physical method of separation that uses a minimum of two immiscible phases to separate chemical compounds based on their relative affinity for each. A two phase system usually consists of an immobile stationary phase and a dynamic mobile

phase which elutes from a column containing the stationary phase. The stationary phase can either be a solid (Cabooter *et al.*, 2011; Beelders *et al.*, 2012a) or liquid (Costa & Leitao, 2011; Costa *et al.*, 2013), while the mobile phase is either a gas, in gas chromatography (GC), a liquid, in liquid chromatography (LC), or a supercritical fluid, in supercritical fluid chromatography (SFC). Gas chromatography is used for the analysis of volatile compounds (Bottcher *et al.*, 2015), whereas LC is used for non-volatile, polar and/or thermally labile analytes. High performance liquid chromatography (HPLC), the modern version of LC, is the most common form of chromatography.

The basic principle of HPLC involves forcing a liquid containing sample components through a column that is packed with polar (normal phase, NP) or non-polar (reversed phase, RP) stationary phases, typically comprising modified silica particles. While the analytes move though the column, molecules of different polarity and molecular weight are separated based on their differential adsorption to the stationary phase. The mobile phase typically comprises mixtures of non-polar solvents for normal phase chromatography and polar solvents for reversed phase chromatography. The mobile phase composition (polarity) can be kept constant as in isocratic analyses, or altered during the analysis in an elution gradient. Altering the mobile phase polarity will change how strongly the molecules adsorb to the stationary phase and thereby allow separation of a much wider range of analytes in a single analysis.

2.2. Sample Preparation

Prior to chromatographic method development, the nature of the sample should be well understood. The sample characteristics will help the chromatographer develop an initial experiment that will generate more meaningful data for further method optimisation (Snyder *et al.*, 1997b).

Samples to be analysed commonly require pre-treatment before analysis. This treatment can involve simple dilution or complex extractions followed by further purification. Pre-treatment is necessary to ensure that interfering molecules or sample constituents harmful to the column are removed. The samples must be homogeneous and the pre-treatment procedure should be reproducible to ensure success of the method (Snyder *et al.*, 1997b).

As rooibos plant material and the analytes of interest in this study are heat, pH and oxygen sensitive polyphenols, prolonged exposure to high temperatures should be avoided when preparing the sample. The sample matrix of the rooibos extract should not interfere with the detection process as this can affect the accuracy of the results. Extracts should be filtered, with the pore size of the filter determined by the particle size of the column to

prevent potential blockage. Care should also be taken to ensure that the sample solvent is compatible with the filter material (Snyder *et al.*, 1997b).

2.3. Stationary Phase

The majority of HPLC columns make use of spherical silica particles as the stationary phase. These silica particles can be produced in various sizes, depending on the specifications the column needs to fulfil. Porous silica particles are typically used and can be produced with narrow internal pores of a few nanometers (nm), where the particle itself is sized in micrometers (µm). An important advantage of silica particles is their high mechanical strength. This property allows a column to be operated at very high pressures without damage to the stationary phase. Furthermore, silica stationary phases in general provide high chromatographic efficiency compared to alternative phases such as graphitised carbon. Silica is also compatible with water and various other organic solvents. For these reasons, silica stationary phases are by far the most common in modern HPLC and extensive knowledge and experience has been gained on these phases, unlike some of the alternative inorganic stationary phases. A disadvantage of silica is that these phases cannot be used at a pH above 8, as silica dissolves at high pH (Snyder *et al.*, 1997b).

Importantly, silica particles can be chemically modified to give the stationary phase a variety of different properties. For the manufacturing of RP stationary phases, silica is derivatised with apolar moieties such as octadecyl (C_{18}) and octyl (C_8) groups (Cavazzini *et al.*, 2011). Longer hydrocarbon chains results in increased retention and higher column capacity.

Currently two column packing types, namely core-shell particles and totally porous particles are mainly used in HPLC. Core-shell particles contain a solid core surrounded by a layer of porous silica stationary phase. The main beneficial property of these phases is that similar performance is obtained to that of smaller fully porous phases. This implies that similar performance is provided at lower back pressures compared to totally porous particles (Hayes *et al.*, 2014), because of increased permeability due to the larger particles (González-Ruiz *et al.*, 2015). Core-shell columns' improved performance are a consequence of lower eddy dispersion, longitudinal diffusion and resistance to mass transfer contributions to band broadening compared to totally porous columns with the same particle sizes (González-Ruiz *et al.*, 2015).

A practical benefit of using core-shell columns is that the lower backpressure (in comparison to a totally porous column of the same particle size) will allow the option of increasing the length of the column, thereby increasing the column efficiency and resolution (R_s). A drawback of core-shell columns is the lower sample loading capacity, a consequence of a reduced amount of stationary phase area being available to react with the sample

compounds (González-Ruiz *et al.*, 2015). Usually the lower loading capacity is not a problem for analytical HPLC, but is problematic for preparative HPLC.

2.4. Mobile Phase

When using RP-LC, the stationary phase usually consists of non-polar groups such as C_{18} or C_8 bound to silica particles, and the mobile phase has a higher polarity. RP-LC mobile phases typically consist of mixtures of water and organic modifiers such as acetonitrile or methanol (Qiao *et al.*, 2011; Beelders *et al.*, 2012b).

By increasing the relative proportion of the organic modifier(s), the mobile phase polarity will decrease, and the retention of compounds will decrease. This means that the elution strength of RP-LC mobile phases increase with increasing content of organic modifier (Baeza-Baeza *et al.*, 2012).

Analysis of multiple compounds with a range of retention properties is generally problematic, especially when using isocratic elution. Under such conditions, separation of either weakly retained or highly retained analytes can be optimised, using weak and strong eluents respectively, but no mobile phase composition will provide optimal separation of all compounds. This is known as the general elution problem (Sternson, 1983). Gradient elutions for RP-LC should start by utilising weak eluents, thereby increasing the elution time of fast eluting compounds (polar compounds in RP-LC). Increasing the elution time of the early eluting compounds should allow increased separation as the most polar compounds will elute first followed by slightly less polar compounds. If the eluent is too strong, multiple compounds will elute simultaneously. The weak eluent's strength should be systematically increased by decreasing the polarity, which will allow less polar compounds to elute at a reasonable time with limited co-elution of compounds with similar polarity ranges. With regards to LC-MS, gradient elutions will not result in significant ion suppression (Ion suppression discussed in more detail later) as interfering salts and water soluble compounds present during injections will elute with the solvent front unlike in the case of isocratic elution, where the presence of these impurities are not removed at the start of analysis (Romanyshyn *et al.*, 2001). Reduced detection interference when utilising gradient elutions compared to isocratic elutions have been observed by Romanyshyn *et al.*, (2001) as better separation can be achieved by gradient elution.

It is possible to theoretically determine the mobile phase composition to gain the best separation after a given column's selectivity has been sufficiently characterised by using the linear solvent strength (LSS) model (Kaliszan *et al.*, 2003). The LLS model can be used in conjunction with quantitative structure-retention relationships (QSRR) to provide approximate elution predictions for structurally defined compounds (Bączek & Kaliszan, 2002).

2.5. Selectivity in HPLC

Selectivity in chromatography is the capacity of a specific separation to differentially retain compounds. Therefore it is a measure of the relative retention of two analytes:

$$\alpha = \frac{k_2}{k_1} \quad (1)$$

Where:

α = selectivity

k_1 and k_2= retention factors for two compounds

Selectivity can be modified in various ways, such as changing the properties of the silica support, changing the mobile phase composition (solvent strength, additives or pH) or changing the analysis temperature.

During the development stages of a new LC method, it is best to initially do a literature search on the sample of interest and acquire a LC method with similar separation goals. By using the same stationary-and mobile phase, replicate the acquired LC method's separation. From this point one can change the mobile phase composition of the analyses to improve the separation if needed. Finally, the column temperature can be modified to finely manipulate the selectivity.

By changing the column temperature, the retention factors (k) of compound are altered, and temperature therefore provides a means of affecting the selectivity factor (α) (see **equation 1**). As a last resort the stationary phase can be changed if all other above mentioned methods failed to deliver the desired separation of the target compounds. If a new stationary phase is selected, mobile phase composition and temperature need to be optimised again.

Chromatographic parameters that were deemed suitable for a given application might not remain ideal as manufacturing of columns evolves. Manufacturing processes and/or the starting silica material can vary as a function of time (Snyder *et al.*, 1997b).

2.6. Efficiency in HPLC

The number of theoretical plates, commonly referred to as the plate number (N), is an indication of column efficiency, i.e. the peak sharpness. Higher plate numbers will give better resolution. The efficiency of the column is determined by its properties such as particle size, packing efficiency and length, operating conditions such as flow rate and temperature and

external factors such as system volume. The simplest way to increase the number of theoretical plates is to increase the column length (Snyder *et al.*, 1997a).

$$H = N/L \tag{2}$$

Where:

H = plate height (mm)

N = number of theoretical plates

L = column length (mm)

One should, however, keep in mind that by increasing the length of a column, the pressure also increases (**equation 3**). Another way to increase the N is by decreasing the diameter of the solid support, but in doing so the pressure will also increase due to lower permeability of the packed bed (Snyder *et al.*, 1997b; Snyder *et al.*, 1997a).

$$p \approx \frac{250 L \eta F}{{d_p}^2 \, {d_c}^2} \tag{3}$$

Where:

p = pressure drop across the column (bar)

L = column length (mm)

η = viscosity of mobile phase (cP)

F = flow rate ($mL.min^{-1}$)

d_p = particle diameter (µm)

d_c = column diameter (mm)

2.7. Resolution in HPLC

The resolution of chromatographic peaks is a measure of how well they are separated. A resolution of 1.5 or higher implies that two peaks are entirely separated. Resolution is dependent on the column efficiency, the selectivity and analyte retention, according to Zhang *et al.*, (1993).

$$\boldsymbol{R_s = 0.25\sqrt{N}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k_2}\right)} \tag{4}$$

Where:

R_s = resolution

N = number of theoretical plates

α = selectivity

k_2 = retention factor of the second compound

As seen in **equation 4**, k does not influence R_s by a large degree for well retained analytes (k>5). The theoretical plate number (N) has a more prominent effect on R_s, but changing N typically requires changing the column dimensions, for which implications such as pressure should be considered. The α term has the largest influence on R_s as a slight increase in α will result in a significant increase in R_s.

3. Countercurrent Chromatography (CCC)

3.1. Introduction

CCC is a liquid chromatographic technique that uses a liquid stationary phase, in contrast to HPLC where a solid stationary phase support is used, and a liquid mobile phase. This technique requires that the analytes of interest are soluble in both phases. The separation principle is based on partitioning of the analytes between two immiscible liquids operating respectively as stationary and mobile phases (Berthod, 2007). One of the liquid phases is kept stationary in a hydrostatic or hydrodynamic column (principles will be explained in the next section). When the sample is injected into the column, the sample compounds will interact with both the liquid phases. Compounds with a high affinity for the mobile phase will be partitioned in the mobile phase and elute more rapidly than compounds with a higher affinity for the stationary phase that in turn will preferentially partition into the stationary phase. Unlike HPLC, compounds with a high affinity for the stationary phase cannot irreversibly bind to the stationary phase and can in theory be completely recovered.

Despite the name, CCC does not necessarily involve two liquid phases that are undergoing countercurrent flow. Rather, the name CCC stems from the fact that the stationary and mobile phase roles can be swapped during an analysis. The name CCC originated from its inventor Yoichiro Ito, who named the technique after countercurrent liquid-liquid distribution apparatus (Berthod, 2007). Usually in chromatography using solid support phases, such as HPLC, late eluting compounds take a long time to leave the column unless gradient elution is used and the column is washed with a strong eluent. Therefore compounds with high retention times usually undergo excessive band broadening inside the column if an isocratic elution is used. Countercurrent chromatography however does not have this problem, because the mobile and stationary phase roles can be interchanged. When interchanging the stationary and mobile phase, firstly inverse “the head to tail”/“tail to head” configuration and then pump stationary phase through the column instead of mobile

phase. This will result in the stationary phase acting as the “new” mobile phase and the “old” mobile phase becoming the stationary phase. Compounds still retained in the column will have a strong affinity of the “new” mobile phase and elute within a reasonable time, compared to the old configuration’s extended elution time.

Countercurrent chromatography has a further important practical advantage. The technique is characterised by a larger loading capacity than most chromatographic techniques, because a higher stationary phase volume is available for interaction with the sample compounds. However, CCC, being mainly a preparative technique, has certain disadvantages such as the fact that it cannot be used to analyse complex samples to such a detailed extent as HPLC. Countercurrent chromatography has lower efficiency compared to HPLC and takes considerably longer to achieve the same quality of separation for complex samples (Berthod *et al.*, 2009).

3.2. Hydrostatic and Hydrodynamic CCC Columns

Countercurrent chromatography can be performed using hydrodynamic or hydrostatic CCC instruments. The characteristics that define a hydrodynamic CCC instrument are spools that contain a coiled Teflon tube, the lack of rotary seals and a gear assembly with two rotation axes to generate the centrifugal field (**Figure 2.4B**). As the columns are rotated, phase decantation occurs when the centrifugal field is high, and when the field direction reverses, the liquid phases form an emulsion where sample compounds can be partitioned between phases. This mixing and decanting process occurs two times in a single rotation and throughout the analysis the phases are pushed to one side of the Teflon coil, referred to as the “head”. The least dense phase will always be on top of the denser phase and this determines in which direction the mobile phase must be pumped. The least dense phase should be pumped from the tail (bottom) end of the column toward the head (top), and the denser phase should be pumped from the head to the tail of the column.

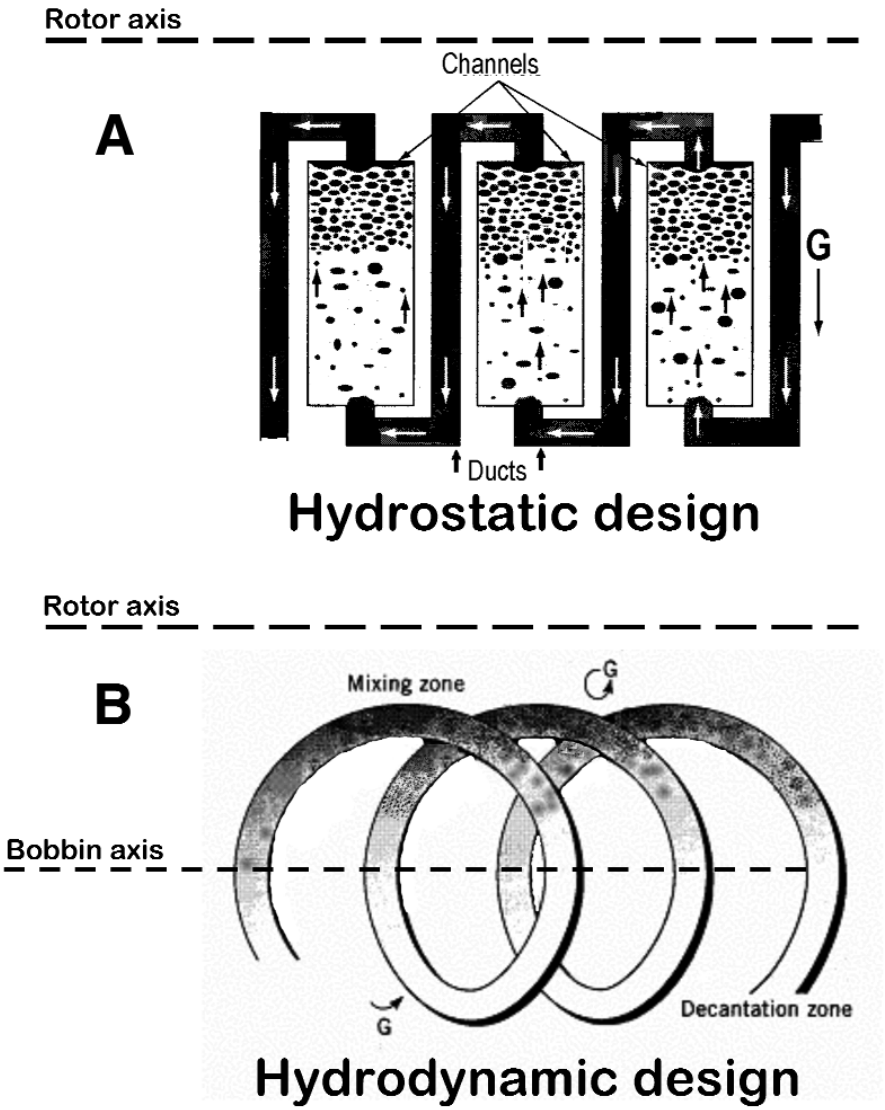


Figure 2.4 The schematic overview of hydrostatic (ascending mode) (A) and hydrodynamic (head-to-tail) (B) CCC columns, illustrated with white representing the stationary phase and black the mobile phase.

The hydrostatic device has its own two unique characteristics (**Figure 2.4A**), the first being repeating geometrical volumes, tubes and channels in which the phases of the solvent system make contact and mix. Secondly, a single axis of rotation allows high rotational speeds and in doing so generates high gravitational forces in order to keep the stationary phase immobile and contained in the column. In contrast to hydrodynamic CCC, in hydrostatic CCC instruments the mobile phase is contained in the connecting ducts and this generates a small hydrostatic pressure, which explains the significant pressure drop needed to contain the stationary phase in the hydrostatic instrument. If the hydrostatic pressure is too high, the mobile phases will lose fluidity, thereby causing an increase in pressure as more energy is needed to pump the mobile phase through the column (Berthod *et al.*, 2009).

As the stationary phase must remain stationary, the mobile phase is the only phase being pumped into the column during elution. If the mobile phase is the denser phase it should enter from the top (head) of the column channels, where it will then form an emulsion with the stationary phase and exit the column at the bottom (tail) duct. This is called the descending mode. The ascending mode is when the mobile phase enters from the bottom (tail) and leaves at the top (head) (Berthod *et al.*, 2009).

3.3. Solvent System Selection

As CCC uses a biphasic liquid, the mobile and stationary phases are cumulatively referred to as the solvent system. Both phases in the solvent system affect each other, although the two liquids are immiscible, small amounts of each dissolve into the opposite phase to the point of saturation (Berthod, 2002a). Due to this, even a small change in the composition of one phase can affect the other phase. To accommodate this, both phases should be prepared together (Berthod, 2007).

The only restriction applicable in the selection of the solvent systems is that the resulting mixture must have at least two phases. This gives the operator a significant amount of freedom in the selection of solvents, but also complicates the process of obtaining the correct solvent system in a timely manner. Due to the almost limitless number of solvent combinations that can be used, it is often not possible to attain an optimal solvent system with a limited number of attempts. One of the best ways to obtain a suitable solvent system is through a literature search (Berthod, 2002a). Examples of comprehensive reviews reporting suitable solvent systems used in literature are Garrard *et al.*, (2007), Costa & Leitao, (2011), Costa & Leitão, (2010), Liu *et al.*, (2015b) and Skalicka-Wozniak & Garrard, (2015). These five studies focus on efficient strategies for selection of a solvent system and reduction of solvent wastage.

The ideal solvent system should have a reasonable distribution-coefficient (K_D) for the target compounds. K_D is a measure of the partitioning of a compound between the two immiscible phases. A low K_D value implies that a compound has a high affinity for the mobile phase, whereas a high K_D value represents a compound that has a higher affinity for the stationary phase, see **equation 5** (Friesen & Pauli, 2007). Ideally, the compounds of interest should have K_D values between 0.25 and 16. Furthermore, the settling time of the solvent system should be less than 30 s as this will result in better stationary phase retention (Costa & Leitão, 2010).

$$K_D = \frac{C_{SP}}{C_{MP}} \quad (5)$$

Where:

K_D = distribution coefficient

C_{SP} = concentration of compound partitioned to stationary phase

C_{MP} = concentration of compound partitioned to mobile phase

Solvent systems with three components can be depicted using ternary phase diagrams. A ternary phase diagram is a 2D graph depicting liquid-liquid phase equilibria in an equilateral triangle for solvent systems consisting of three solvents with solvent amounts as volume fractions (see **Figure 2.5**). The orthogonal representation specifies the concentration of only two solvents and the third can be calculated since the sum of the three amounts is 1. Solvent systems can be grouped into “types” based on their characteristics, with the most common types termed type 0, 1 and 2. Type 0 solvent systems consist of three solvents where each combination of the solvents is miscible, but for certain ternary compositions two phases are formed. Type 1 and 2 systems consist of two immiscible solvents with a third that is miscible with both or one of the other solvents, respectively.

One way to choose a solvent system is by using the “best solvent” approach. This is done by choosing a good solvent (the “best solvent”) for the sample (i.e. one in which the analytes of interest are highly soluble) and two other solvents that are immiscible with each other. The “best solvent” should be able to partition between the other two solvents, thereby leading to convenient K_D values for the compounds of interest. This approach can be used with type 0 and 1 solvent systems.

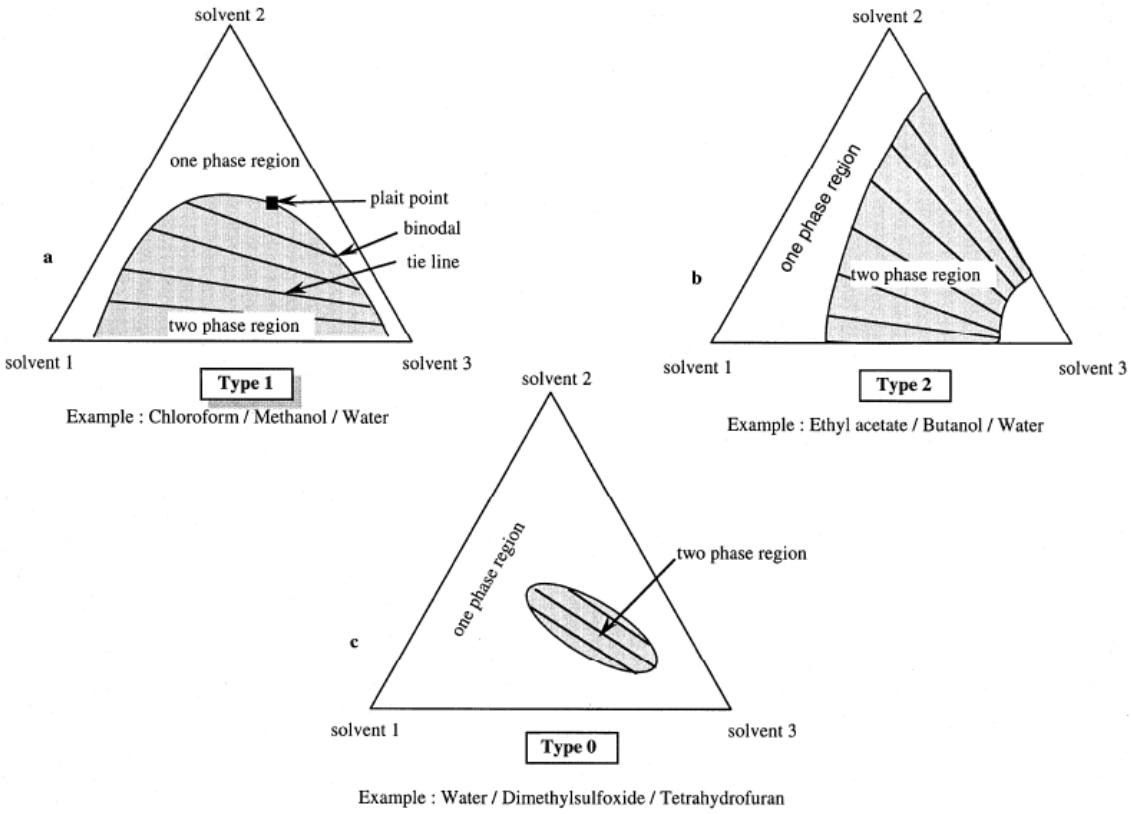


Figure 2.5 Visual representations of ternary diagrams for the three most used type of solvent systems in CCC (Foucault & Chevolot, 1998).

Another way to choose a solvent system is via the multisolvent system approach, where solvent systems are grouped into solvent system families. With different solvent systems combining differing ratios of the same set of solvents resulting in a stepwise increase in average polarity of the solvent systems.

An example of a solvent system family is the “ARIZONA” solvent system family (**Table 2.2**). The ARIZONA approach combines two binary systems, namely solvent system A (polar system) and solvent system Z (non-polar system) (Berthod, 2002a; Berthod *et al.*, 2005).

Table 2.2 Composition of ARIZONA system's multiple solvent systems (v.v^{-1})

ARIZONA nr	Heptane	Ethyl acetate	Methanol	Water
A	0	1	0	1
B	1	19	1	19
C	1	9	1	9
D	1	6	1	6
F	1	5	1	5
G	1	4	1	4
H	1	3	1	3
J	2	5	2	5
K	1	2	1	2
L	2	3	2	3
M	5	6	5	6
N	1	1	1	1
P	6	5	6	5
Q	3	2	3	2
R	2	1	2	1
S	5	2	5	2
T	3	1	3	1
U	4	1	4	1
V	5	1	5	1
W	6	1	6	1
X	9	1	9	1
Y	19	1	19	1
Z	1	0	1	0

A phenomenon that might complicate solvent system selection for a hydrodynamic instrument is the formation of emulsions between the mobile and stationary phases due to emulsifying properties of sample components. An emulsion is when a liquid is suspended as droplets in another immiscible liquid (Lam & Nickerson, 2013). This is an unwanted effect as the sample components must be able to freely partition between the two phases of the solvent system. Emulsion formation may lead to stationary phase loss, which may decrease compound separation or completely displace the stationary phase, resulting in no separation.

3.4. Stationary Phase Retention

When a liquid stationary phase is used, the first question that comes to mind is how the stationary phase remains stationary. This is achieved by rotating the column tubing at a high number of revolutions. The liquid stationary phase will be held immobile due to gravitational forces that are generated by rotation of the column. By increasing the instrumental rotational speed around the axis, stationary phase retention is facilitated as higher gravitational forces are generated (Berthod, 2002a).

The stationary phase, being liquid, is subjected to loss during analysis. The major cause of stationary phase loss is when the mobile phase is introduced into the column, which pushes out some of the stationary phase. Stationary phase retention (S_F) is defined according to **equation 6**:

$$S_F = \frac{V_S}{V_M} \tag{6}$$

Where:

V_S = volume stationary phase

V_M = volume mobile phase

The higher the flow rate of the mobile phase, the more stationary phase will be lost (Berthod, 2002b). For this reason the flow rate should be kept as low as possible keeping time constraints in mind. In order to allow effective separation, a minimum of 10% stationary phase must be retained (Berthod, 2002a). Reduction in stationary phase retention also reduces the retention factor, selectivity and resolution of the column as explained later (Berthod, 2002a).

3.5. Selectivity and Resolution in CCC

CCC offers a near infinite number of solvent system combinations to fine-tune the selectivity by adjusting the solvent system. The main problem with regard to CCC selectivity is when the stationary phase is not efficiently retained. Increasing the mobile phase flow rate or decreasing the column's rotational speed will result in increased stationary phase loss. As the stationary phase is lost, retention factors will decrease. Another parameter that must be regulated is temperature. As the temperature of the solvent system increases, the mutual solubility of the liquids in the two phase system increases. Therefore changing the temperature will result in changes in the composition of the two phases, as a varying amount of each phase will dissolve into the other (Berthod, 2002a).

Resolution (R_s) in CCC (**equation 7**), like in chromatography (*cf.* **equation 4**) relies on three aspects, namely efficiency (the number of theoretical plates), selectivity and retention.

$$R_S = S_F \frac{\sqrt{N}}{4} \frac{(K_{D2} - K_{D1})}{(1 - S_F\left[1 - \frac{K_{D2} + K_{D1}}{2}\right])} \quad (7)$$

Where:

N = number of theoretical plates

K_{D1} and K_{D2} = distribution coefficient of two compounds

Equation 7 implies that resolution is dependent on how effectively stationary phase is retained. Usually in HPLC the focus is on increasing the efficiency to increase the resolution as columns with solid support phases do not have as large an area in which the sample compounds can react with the support phase as columns without solid support phases such as CCC. In CCC the efficiency is generally low, but good resolution can nevertheless be obtained due to the high amount of stationary phase available for interaction with the sample compounds (Berthod, 2002a).

3.6. Elution-Extrusion

In CCC, elution-extrusion refers to a technique that can be used to reduce the analysis time. This is performed by operating in either reversed or normal phase mode for a time (generally using two column volumes of mobile phase), and then extruding the entire content of the column by pumping stationary phase instead of mobile phase. The extrusion will push out all the stationary phase, including compounds that have already been separated inside the column. This will decrease both the analysis time and band broadening, as the compounds will have less time to disperse in the solvent system (Berthod *et al.*, 2009). The difficult part of this approach is to determine when to start the extrusion process, as premature extrusion will result in compounds not being completely separated and extruding too late will result in band broadening and wasted time. It is possible to theoretically determine when to start the column extrusion by using the distribution coefficients (K_D) of the sample components to determine when compounds will be separated from each other, or alternatively a trial-and-error approach can be used. **Figure 2.6** provides a visual representation of the elution-extrusion process.

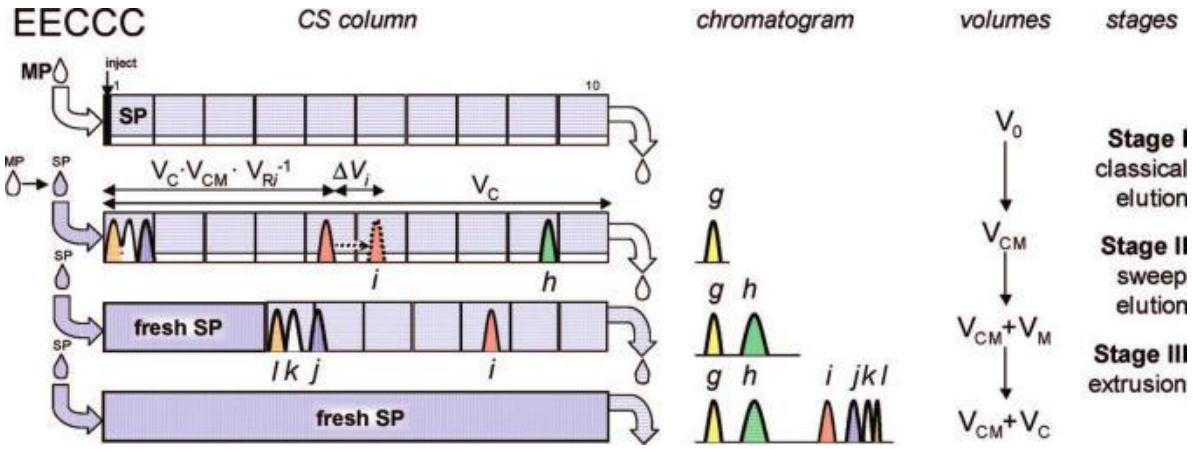


Figure 2.6 Illustration of the elution-extrusion method used in CCC. Band broadening is diminished for the compounds that have been extruded as opposed to compounds g and h that eluted before the extrusion step (Pauli *et al.*, 2008).

3.7. Gradient Elution in CCC

To change the strength of the mobile phase during chromatographic analysis is very beneficial as analytes of a wide range of polarities can be analysed within a reasonable time. This concept is ideal for HPLC, where the stationary phase is not significantly affected by the composition of the mobile phase, but for CCC additional complications have to be considered. As the mobile phase strength increases, the stationary phase composition will change as some of the mobile phase will dissolve into the stationary phase. Furthermore, a change in the density of either of the solvent system's respective phases might occur as the composition of the phases change throughout the gradient elution. If the density change of a single phase is too severe, the density of the upper phase might become higher than that of the lower phase, thereby ruining the column due to the loss of stationary phase.

To prevent ruining the column, the solvent that partitions best between the immiscible phases should be selected as the gradient solvent. The gradient solvent will be the solvent responsible for loss of stationary phase during the analysis as the amount of gradient solvent changes (Costa *et al.*, 2013). Ignatova *et al.*, (2011) proposed a method for estimation of the best solvents and solvent composition for gradient elution. The estimation was based on measurement of initial and final stationary phase retention for a gradient elution run, calculating the K_D obtained during the gradient run and determining the best HEMWat (a solvent system family consisting of mixtures of *n*-hexane/ethyl acetate/methanol/water) (Friesen & Pauli, 2005) solvent system from a correlation of the static K_D against the HEMWat solvent system number. The authors then used this information to predict how the solvent system should be altered to provide better resolution and separation.

Gradient elution is not common in CCC. From 1984 to the end of 2014, 2594 solvent systems have been described, of which only 272 were gradient systems (Skalicka-Wozniak & Garrard, 2015).

4. Chromatographic Detection Methods

4.1. Introduction

Using the appropriate detection methods for polyphenolic compounds separated by HPLC/CCC is crucial. Various detectors can be used for LC analysis of phenolics, as reviewed by De Villiers *et al.*, (2016), namely electrochemical detection (ED), fluorescence (FL), UV-vis, diode array, nuclear magnetic resonance (NMR) and MS detectors. The most common detectors for rooibos polyphenols, UV-vis and MS (Beelders *et al.*, 2012a; Beelders *et al.*, 2012b; De Beer *et al.*, 2015), will be discussed in further detail.

4.2. UV/Vis

Diode array detectors (DAD), which are capable of simultaneously detecting absorbance across the UV-Vis part of the electromagnetic spectrum (190-750 nm), are commonly used in the analysis of polyphenolic compounds. The majority of the known polyphenolic subclasses can be detected and differentiated based on their UV-Vis spectra, thereby allowing one to distinguish between different subclasses. In cases where phenolic compounds have near identical MS spectra, but belong to different phenolic classes (for example dihydrochalcones and flavanones), they can be distinguished based on their UV-Vis absorption spectra (Xu *et al.*, 2013).

For flavonoids present in rooibos, UV detection is usually performed at around 288 nm for dihydrochalcones, phenylpropanoids and flavanones, and 350 nm for flavonols and flavones (Joubert, 1996; Beelders *et al.*, 2012b). UV-Vis detection sensitivity varies depending on the polyphenolic compound being analysed, but is generally sufficient for quantity of polyphenolic compounds present in natural products (De Villiers *et al.*, 2016).

An advantage of DAD is that this detector can be hyphenated to conventional HPLC or CCC instruments, preferable directly after the column as it is a non-destructive detector. UV/Vis detectors are compatible with the relatively high mobile phase flow rates HPLC (Lin & Harnly, 2012). It has been noted that unfavourable conditions during HPLC and CCC analyses can lead to the formation of air bubbles in the mobile phase (usually when an organic phase is rapidly introduced to an aqueous phase), which leads to increased noise (Oka & Ito, 1989). Liquid phases can be sonicated before use to degas them to overcome this occurrence (Sasikiran Goud *et al.*, 2014). Modern HPLC instruments generally have on-line degassing units for this reason.

Compared to HPLC, CCC has higher potential to produce a high noise with UV-Vis detection (Oka & Ito, 1989). This can be as a result of steady carryover of stationary phase due to improper choice of elution mode or high mobile phase flow rates. Migration of stationary phase into the flow cell, caused by variations in rotational speed, sample overloading or vibration of the centrifuge system can also cause high noise. These problems can be mitigated by applying better experimental conditions (Oka & Ito, 1989). Turbidity of thermolabile mobile phases due to increased ambient temperatures and bubble formation of the effluent under reduced pressure in the periphery of the flow passage are also problems that lead to increased noise. These problems can be overcome by selecting a suitable UV-Vis monitoring system such as inserting a fine PTFE tube between the column outlet and detector and submerging the tube in thermostatted water. A PTFE tube of similar dimensions should be added to the effluent from the UV-Vis detector to create back pressure to reduce bubble formation (Oka & Ito, 1989; Drogue *et al.*, 1991).

4.3. Mass Spectrometry (MS)

Mass spectrometry (MS) is currently the most powerful detector for use in combination with chromatography. In MS, the mass of ions are measured. The basic steps of MS involve (1) generation of ions, (2) separation of ions based on their mass-to-charge ratios *(m/z)* using a mass analyser and (3) detection of the ions (Gross, 2004). The most important advantages of MS are that only a small amount of sample is needed to yield significant analytical data, and information suitable for structural elucidation can be obtained. MS is a destructive technique, which implies that the MS should be the last detector in multi-detector systems (Gross, 2004).

An MS instrument consists of a sample inlet, an ion source, a mass analyser and a detector. Electrospray ionisation (ESI) is the most common ionisation technique for LC-MS, especially when studying polyphenols. The mass analyser and detector are subjected to high vacuum conditions during analysis, while the ion source is at atmospheric pressure for LC-MS analyses. The high vacuum reduces the particle density inside the mass spectrometer and so allows control of the number of collisions the ions are involved in. This effect is especially important for high resolution mass analysers such as time-of-flight (TOF) systems. The vacuum also assists in accelerating the ions from the sample inlet to the detector. Electron multipliers, which are typically used as detectors, are sensitive to air as the high voltage can cause arcing and the emissive layer is sensitive to the presence of air (Gross, 2004).

Mass spectrometry is currently one of the most sensitive methods of detection methods that can be used in combination with HPLC (Holcapek *et al.*, 2012). The field is also constantly evolving, with new instrumentation providing better resolving power and

sensitivity. LC-MS can be used to analyse polyphenols in positive or negative ionisation modes (see below). Characteristic MS fragmentation patterns of polyphenols in conjunction with UV-Vis spectral information can be used to tentatively identify polyphenolic compounds. It should be noted that MS cannot distinguish between molecular isomers and therefore such compounds should preferably be separated before tentative identification can be attempted (Forcisi *et al.*, 2013).

Early experiments aimed at coupling HSCCC with ESI-MS using a frit-MS system to analyse biological samples proved the feasibility of this combination (Oka *et al.*, 1991). The system's performance was evaluated by injecting and separating three indole auxin mixtures, two mycinamicin mixtures and a colistin complex. Jerz *et al.*, (2010) directly coupled ion-pair HSCCC with ESI-MS for the analysis of betacyanins in *Bougainvillea glabra*. The HSCCC flow rate was 3 $mL.min^{-1}$, which was adjusted by a low pressure split-unit to 10 $\mu L.min^{-1}$ (1:325 split ratio) for continuous ESI-MS/MS detection

4.3.1. Electrospray Ionisation

Electrospray ionisation is a soft ionisation technique that is most often used in the hyphenation of HPLC and MS. The most important requirement in this process is that analytes in solution are charged and transferred to the gas phase. Electrospray ionisation is also most often used for the ionisation of polyphenols. This technique is the "softest" of all the soft ionisation techniques as the sample undergoes very little fragmentation during ionisation, with the molecular ion commonly being predominant. A disadvantage of ESI is that structural information of the sample compound is limited to molecular weight (MW) information, but to counter this, ESI can be used in combination with in-source fragmentation or tandem MS techniques (Gross, 2004).

In ESI, a liquid sample at atmospheric pressure is sprayed from a capillary at high potential difference into the ionisation chamber; the strong electrostatic forces and nebulising gas flow result in the formation of a fine spray of ionised droplets. Warm nitrogen gas is fed in the opposite direction to the spray, which facilitates evaporation of the solvent in the droplets. As the solvent evaporates and the charge density on the surface of the ion droplets grow, the Rayleigh limit will be reached, at which point the droplet will disintegrate into smaller ion droplets. This process is known as the droplet jet fission (Gross, 2004). Gas phase ions are then produced from these small charged droplets in one of two ways. The first is termed the charged residue model (CRM) theory, which proposes that complete desolvation occurs, resulting in single analyte ions. The second theory is named the ion evaporation model (IEM). The IEM assumes that direct evaporation of solvents occurs from the surface of highly charged droplets. The CRM best describes solvent evaporation for

large molecules and the IEM best describes solvent evaporation for small molecules (Kebarle, 2000). Faster solvent evaporation will result in higher average charged states. Electrospray ionisation is generally only used to generate ions of *m/z* values up to 3000, but can be used up to *m/z* 8500 (Kebarle, 2000). **Figure 2.7** shows a picture of the spray of charged droplets during ESI.

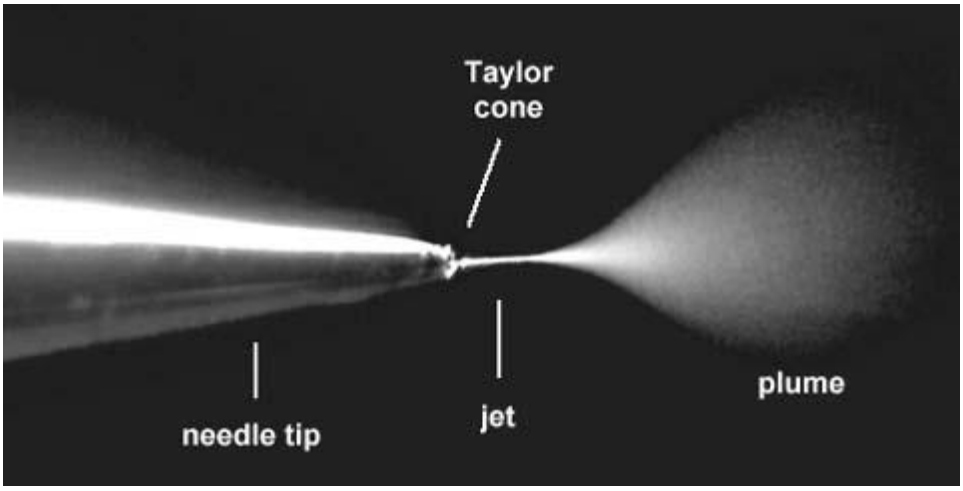


Figure 2.7 Illustration of the spray of droplets formed at the needle tip during electrospray ionisation from a nanocapillary (Gross, 2004).

4.3.2. Ion Suppression

During reversed phase separation ion suppression is necessary as ionised compounds have low retention; ionised compounds are poorly separated by RP-LC. Electrospray ionisation in contrast requires that ions are ionised in solution. If ionisation is suppressed, the result will be reduced sensitivity.

Fortunately MS does not require compounds with different mass spectra to be completely separated in order to be detected and studied, as they can be distinguished based on their mass spectra. The associated disadvantage is potential ion suppression under such conditions. The generally accepted theory is that the majority of ion suppression occurs when matrix compounds compete with analytes during ionisation (ESI) (Furey *et al.*, 2013). Certain mobile phase additives such as trifluoroacetic acid (TFA) can inhibit ionisation in negative mode through ion-pair formation, also affecting subsequent analyses (Faccin *et al.*, 2016). Ion paring reagents such as TFA are used to provide increased retention in RP-LC (Mallet *et al.*, 2004; Kojima *et al.*, 2010).

The mechanism of ion suppression and enhancement is still poorly understood, but mobile phase composition and analyte co-elution have been noted as primary causes of this phenomenon and methods should be designed with this possible disadvantage in mind (Remane *et al.*, 2010; Faccin *et al.*, 2016).

4.3.3. Positive and Negative Ionisation Modes

Analytes can be ionised in positive or negative ionisation modes in ESI. Simply put, in the positive mode molecules are mostly protonated, whereas in negative mode they are deprotonated. This implies that positive ionisation mode is better suited to basic molecules, and negative mode to acidic compounds.

Negative mode has been found to be more sensitive for flavonoids, although less useful fragmentation information may be observed (Cuyckens & Claeys, 2004; Parejo *et al.*, 2004). Both positive and negative ionisation modes are compatible with either NP- or RP-LC. Positive ionisation mode is considered to deliver better structural elucidation than negative ionisation mode, as the fragments that form are easier to interpret due to more structural details provided from the resulting fragment. (Sakushima *et al.*, 1988; Abad-Garcia *et al.*, 2008). Using both positive and negative mode gives extra certainty that the molecular mass and structural data are accurate, especially where the signal to noise ratio is low (Abad-Garcia *et al.*, 2009).

4.3.4. Mass Analysers

The basic purpose of a mass analyser is to separate ions based on their *m/z* values (El-Aneed *et al.*, 2009; Holcapek *et al.*, 2012). Mass analysers are distinguished by their resolving power, which is usually correlated to the cost of the instrument. The resolving power can be defined as the inverse of the smallest resolvable *m/z* value between two peaks (Wollnik, 2015).

Common mass analysers used for LC-MS, in order of increasing resolving power and cost, are quadrupole, ion-trap, TOF, orbitrap and a ion cyclotron resonance (ICR) mass analysers (Gross, 2004; Holcapek *et al.*, 2012). The following sections will focus on the common mass analysers used in this study, the quadrupole and TOF mass analysers.

4.3.4.1. Quadrupole Mass Analysers

A linear quadrupole consists of four cylindrical rods pointed in the Z-direction and mounted in a square configuration relative to each other. A quadrupole mass analyser is shown in **Figure 2.8**. The rods opposite each other have the same potential energy and are connected electrically with direct current (DC) and alternate radio-frequency to affect the trajectory of ions. In basic terms, as the ions traverse the quadrupole in the Z-direction, the field created by the rods will allow only ions of a particular *m/z* ratio through the quadrupole to reach the detector; ions of different *m/z* ratios undergo unstable trajectories and collide with the rods, and are therefore not detected.

The trajectories of the ions are rather complicated, as the ions undergo a complex oscillating flight path. If an ion is positively charged it will move towards a negatively charged

rod, but when the polarity of the rod changes, the ion will be repelled. This oscillating flight path will continue until the ion reaches the detector or collides with a rod. This process allows the accurate selection of ions particular *m/z* values to pass to the detector (El-Aneed *et al.*, 2009).

Quadrupoles can be operated in either scan or selected ion monitoring (SIM) modes. When operating in scan mode, the mass filter is set to scan through a range of masses. This is usually done when the operator does not have prior knowledge about the exact composition of the sample compounds so as to enable identification of compounds based on their mass spectra. The sensitivity is lower than in SIM mode, because the quadrupole only spends a fraction of the scan time acquiring data for each particular *m/z* value (Steen *et al.*, 2001). In SIM mode the mass analyser only acquires data for selected ions. As a result, SIM is characterised by very high selectivity and sensitivity, since the quadrupole spends more time collecting data for fewer ions (Gross, 2004). SIM is therefore useful when quantification of selected target molecules is to be done using MS, but is not suited for the identification of unknown compounds.

Quadrupoles are often combined with additional mass analysers in tandem MS instruments, the most common being triple quadrupole (QqQ) and quadrupole-time-of-flight (Q-TOF) systems. The benefit of combining a quadrupole with a TOF analyser is that the high sensitivity and resolving power of the TOF can be combined with the selectivity of the quadrupole to perform selective collision-induced dissociation (CID, see below) experiments to obtain structural information for particular compounds.

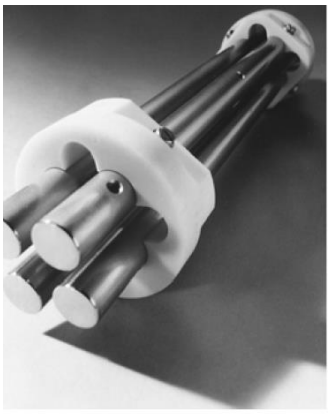


Figure 2.8 Photo of a quadrupole mass analyser (Gross, 2004).

4.3.4.2. Time-of-Flight (TOF) Mass Analysers

In a linear TOF instrument, ions are directed down a field-free flight tube with the same initial kinetic energy. The TOF analyser then uses the relationship between the time that ions take to travel the length of the flight tube and their *m/z* values to determine the mass of each ion

with high accuracy. Ions with higher *m/z* values take longer to cover the same distance (Gross, 2004; De Villiers *et al.*, 2016). The advantage of a TOF above a quadrupole is that theoretically all the ions formed should reach the detector (El-Aneed *et al.*, 2009).

The conventional linear TOF has the disadvantage that ions do not acquire exactly the same kinetic energy, which leads to a small spread of flight times for ions of the same *m/z*, which in turn negatively affect the resolving power. In order to avoid this limitation, a reflectron (electrostatic ion mirror) was designed; **Figure 2.9** presents a visual representation of the differences between a conventional TOF and a TOF with a reflectron. A reflectron is an optic device which can change the flight path of ions entering the linear tube. Ions with larger kinetic energy will penetrate deeper into the reflectron, which result in ions being gradually repelled depending on their respective kinetic energies. The flight path is subsequently changed to a V-shape with a detector at the end of the ion path. The increased flight path has the added advantage of increasing the mass accuracy and resolving power (Mamyrin & Shmikk, 1979).

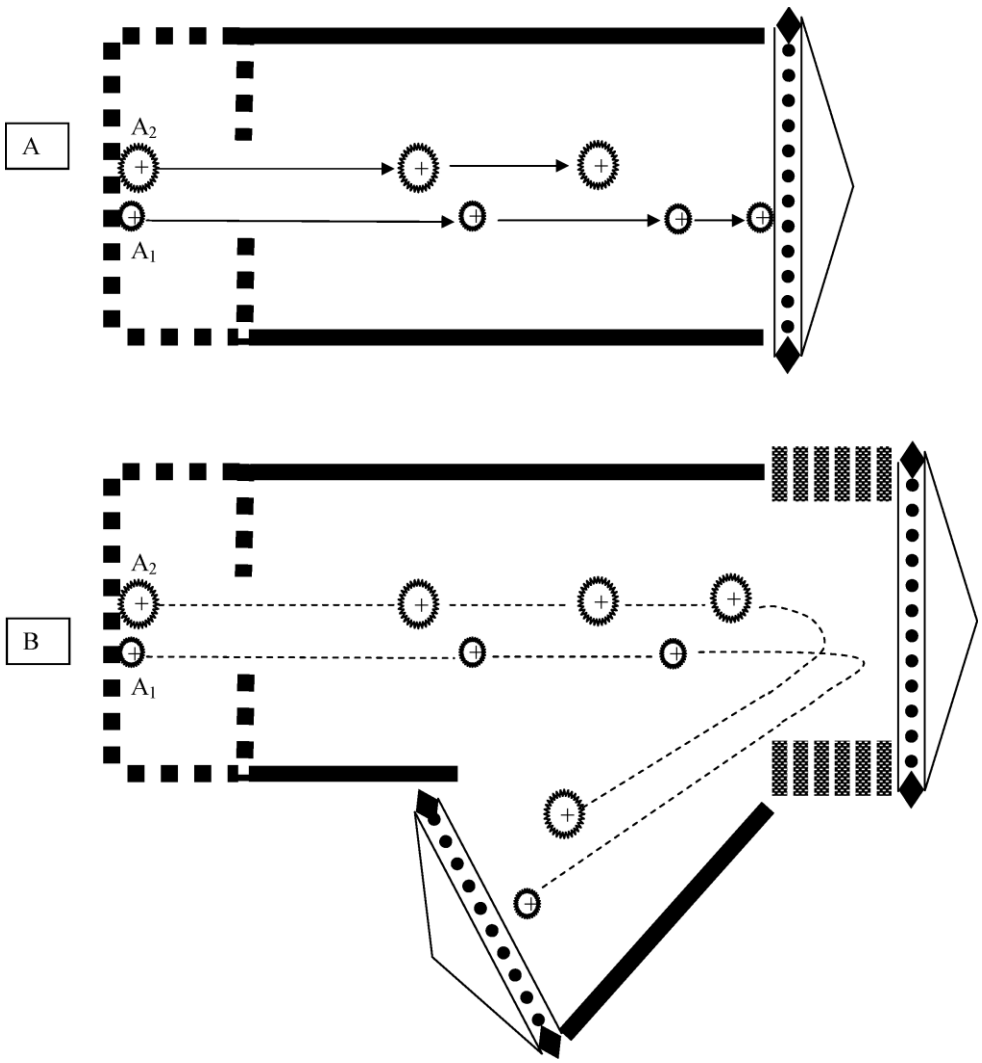


Figure 2.9 A schematic depiction of the difference between a linear TOF mass analyser (A) and a reflectron type TOF mass analyser (B) (El-Aneed *et al.*, 2009).

Time of flight instruments offer very high mass ranges without compromising sensitivity. These instruments are also capable of high speed acquisition and have sufficient accuracy and resolving power to allow assignment of molecular formulae to compounds (Jiwan *et al.*, 2011).

4.3.5. Tandem Mass Spectrometry

In tandem MS (MS/MS) instruments, two or more mass selective operations are performed after the other (March, 1997). Tandem MS can be performed as tandem-in-space, which involves coupling two mass analysers in series to allow the different processes to happen at separate physical locations. In contrast, in tandem-in-time MS/MS experiments such as performed using ion trap instruments, the different processes are carried out in the same space, but at different time intervals (Johnson *et al.*, 1990; Garrido Frenich *et al.*, 2008). This study will further only discuss tandem-in-space techniques as tandem-in-time techniques were not relevant for this study.

The purpose of combining multiple MS stages is to gain additional information by the combination of multiple mass analysis steps. The initial mass analyser typically isolates selected *m/z* values which are then fragmented by CID and the ion fragments are detected by a second mass analyser. For the purpose of this study, Q-TOF systems will be discussed briefly.

The idea of a Q-TOF is to combine the scanning power of a quadrupole with the high resolving power of a TOF analyser in order to generate accurate mass data for selectively fragmented compounds to allow their identification. The quadrupole can isolate selected ions of interest (in SIM mode), which are then fragmented by CID in the collision cell located between the quadrupole and TOF mass analysers. The formed ion fragments are then detected by the TOF analyser with a high mass accuracy (Morris *et al.*, 1996; El-Aneed *et al.*, 2009).

4.3.6. Collision Induced Dissociation (CID)

Collision induced dissociation (CID) is the fragmentation of gaseous ions that were stable before the CID event. This process allows more effective structural elucidation of unknown compounds by providing additional information on their fragmentation. CID can be performed between the ionisation source and the mass analyser in a single-stage MS instrument, or more commonly in a collision cell placed between the mass analysers in a tandem MS instrument. An example of this is the QqQ where the second quadrupole acts as the CID cell (Steen *et al.*, 2001). In the latter case, CID is performed by passing the ion beam through a collision cell with narrow entrance and exit slits. Collision gas, usually nitrogen, is injected into the collision cell where the pressure is considerably higher than the surroundings. The

ions will collide with gas molecules and fragment, allowing detection of these fragmentation ions (Gross, 2004). In high intensity beams, multiple collisions can occur. This means that the compounds will undergo more than one fragmentation step. Ion beams of low energy will lead to less collisions and therefore less fragmentation (Gross, 2004).

4.3.7. Electrospray ionisation (ESI)-MS in the Identification of Polyphenols

For structural elucidation, identification and quantification of polyphenols in natural products, ionisation is most commonly performed by ESI (Abad-Garcia *et al.*, 2008; Ignat *et al.*, 2011). An important trait of ESI is that it is a soft ionisation technique, meaning that minimal internal energy is transmitted to the analytes during ionisation (El-Aneed *et al.*, 2009). This has the advantage of mostly forming the molecular ion; only very weak covalent bonds might be cleaved under these condition (for example usually the *O*-glycosidic bond), which will lead to additional ions being detected. ESI can be used in either the positive or negative modes for phenolic compounds.

Single stage MS is useful to obtain information about the intact molecular ion (either protonated or deprotonated) and ions formed from the fragmentation of *O*-glycoside sugar moieties (Abad-Garcia *et al.*, 2008; Abad-García *et al.*, 2009). If a high resolution instrument, such as a TOF, is used, the molecular formula can be obtained. In order to gain more detailed structural information, tandem MS will need to be performed as limited fragmentation occurs due to the ESI ionisation process (Charles, 2014). Polyphenols of different classes will undergo different fragmentation pathways. For example, dihydrochalcones fragment differently from flavonols of the same molecular formula.

During tandem MS analyses, high collision energy will typically result in cleavage of glycosidic groups, as well as potential fragmentation of the aglycone moieties. In the following discussion on the fragmentation behaviour of flavonoids (Parejo *et al.*, 2004), the carbon and ring numbering for flavonoids shown in **Figure 2.10** will be used.

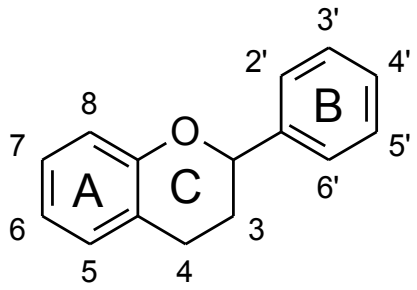


Figure 2.10 Basic structure of a flavonoid aglycone with carbon and ring numbering indicated.

The fragmentation of flavonoid glycosides depends on the nature and position of the glycosidic bond. Flavonoids can have *C*- or *O*-glycosidic bonds. The *C*-glycosidic bond usually occurs at C6 or C8 positions (**Figure 2.10**) (Abad-Garcia *et al.*, 2008). The *O*-glycosidic bond involves a sugar moiety bound to hydroxyl group(s) (Abad-Garcia *et al.*, 2009). *O*-glycosidic bonds are usually weak bonds, and therefore they are cleaved under low collision energy conditions, leading to neutral loss of the sugar moiety. Knowing this, one can tentatively determine which sugar moieties are attached to the flavonoid based on the observed neutral loss (Cuyckens *et al.*, 2001; Abad-Garcia *et al.*, 2008). Rooibos phenolics that contain *O*-glycosidic bonds include glycosylated flavones, flavonols and PPAG (Beelders *et al.*, 2012b). These compounds therefore have simple MS/MS spectra.

In flavonoid-*C*-glycosides, the strong C-C bonds require substantially more energy to fragment. At high collision energies, cross-ring cleavage of the sugar moiety occurs, with associated loss of water and glycosidic methylol groups as formaldehyde (Ferreres *et al.*, 2007; Abad-Garcia *et al.*, 2008). The higher collision energy generates more complex mass spectra than is the case for *O*-glycosidic flavonoids. Further increasing the collision energy can result in cleavage of the C ring of the aglycone moiety. The resultant fragments can be used to study the substitution of the A and B rings (Abad-Garcia *et al.*, 2008).

Based on extensive experimental MS/MS data obtained for flavonoids, general guidelines for the tentative identification of these compounds can be derived. An example is of a general scheme to differentiate between flavonoid-*C*- and *O*-glycosides and characterisation of *C*-glycosides is presented in **Figure 2.11**. **Figure 2.12** shows an example of the proposed fragmentation mechanism of di-*C*-glycosides, using apigenin 6,8-di-*C*-β-D-glucoside as example (Cao *et al.*, 2014). The *C*-glycoside moieties are not cleaved from the aglycone, but rather characteristic (^{0,2}X = neutral loss of 120, ^{0,3}X = neutral loss of 90) cleavages of the sugar moieties are observed. Successive losses of CO and 2CO also occur.

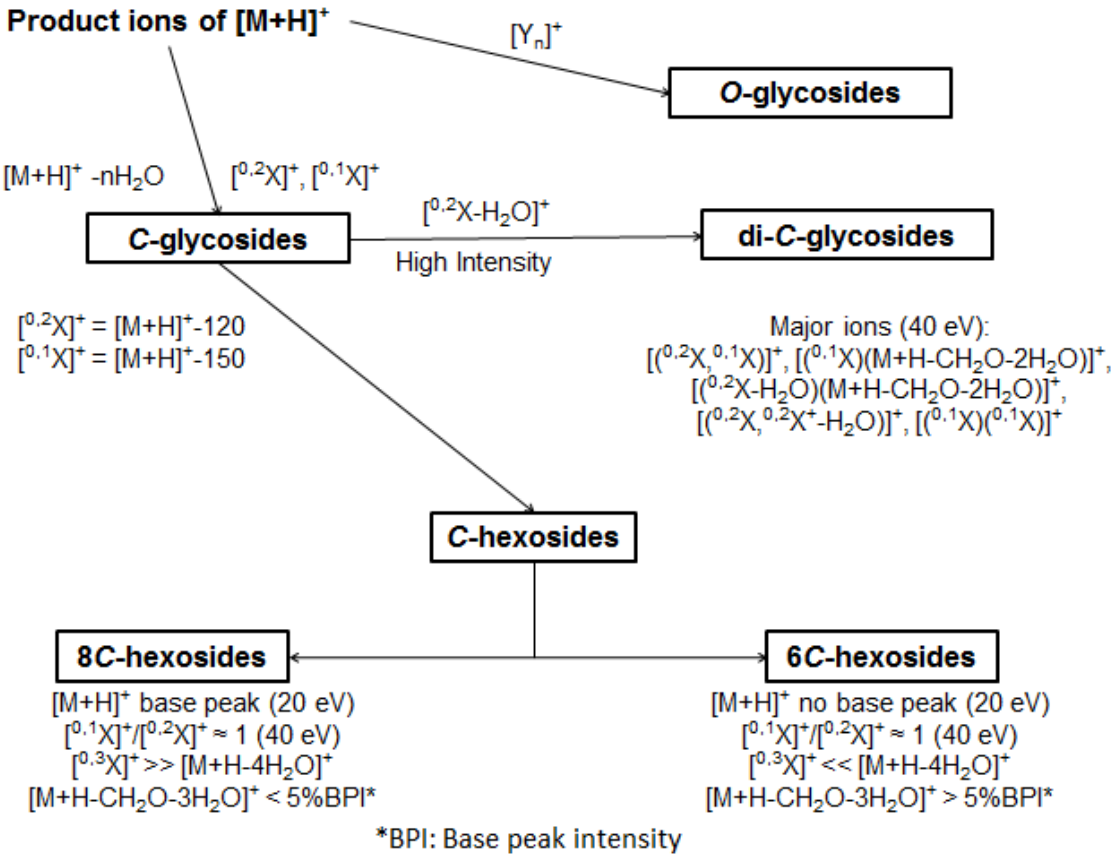


Figure 2.11 Guideline proposed by Abad-Garcia *et al.*, (2008) for characterising flavonoid-glycosides. For interpretation of the fragmentation nomenclature, the reader is referred to Abad-Garcia *et al.*, (2008), Abad-Garcia *et al.*, (2009) and Cao *et al.*, (2014).

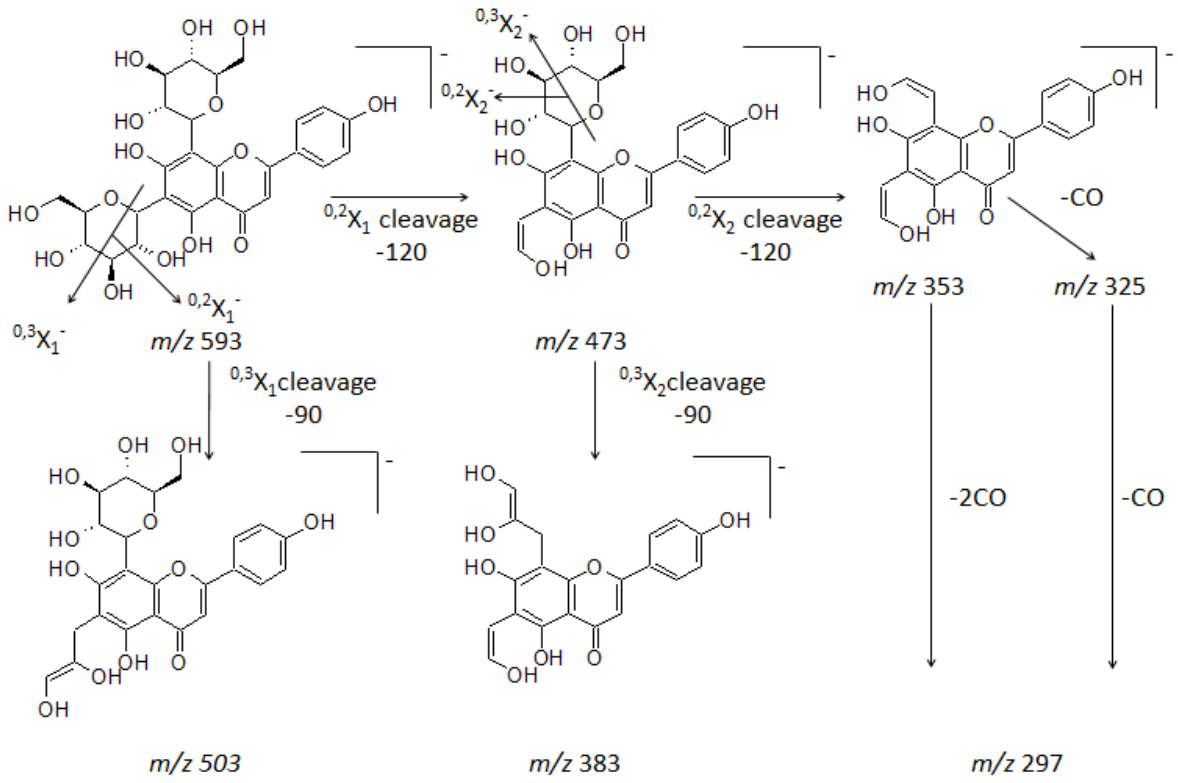


Figure 2.12 Proposed fragmentation pathways of apigenin-6,8-di-*C*-β-D-glucoside in negative ionisation mode (Cao *et al.*, 2014). For interpretation of the fragmentation nomenclature, the reader is referred to Abad-Garcia *et al.*, (2008), Abad-Garcia *et al.*, (2009) and Cao *et al.*, (2014).

5. Two-Dimensional (2D) Chromatography

5.1. Introduction

When complex samples are analysed, one dimensional (1D) chromatography may not provide sufficient resolving power (Fanali *et al.*, 2013). In other words, the performance limit of any specific chromatographic technique is eventually reached when analysing highly complex samples.

Multidimensional separations potentially provide much higher separating power than 1D chromatographic methods. Multidimensional chromatography involves the use of more than one separation step to provide improved separation of complex samples (Kalili & De Villiers, 2013). To exploit the benefits of multidimensional separations, each step should be based on different separation principles, which are referred to as orthogonal separations (see below) (Liu *et al.*, 2009). Examples of separation mechanisms combined for multidimensional separation are RP-LC×RP-LC using two different stationary phases

(Francois *et al.*, 2009a; Mondello *et al.*, 2010), hydrophilic interaction chromatography (HILIC)×RP-LC (Beelders *et al.*, 2012a), ion exchange chromatography (IEX)-LC×RP-LC (Li *et al.*, 2014) or NP-LC×RP-LC (Dugo *et al.*, 2004; Cacciola *et al.*, 2012).

Two-dimensional chromatography can be performed in either heart-cutting or comprehensive modes. In heart-cutting 2D chromatography, single or selected fractions of interest are analysed in the second dimension (^{2}D). This mode is relatively fast and simple to perform (Fanali *et al.*, 2013). Accordingly, heart-cutting 2D chromatography is normally used to provide additional information on selected portions of the original sample. The drawback of this technique is that only a fraction of sample components are analysed in both dimensions, which implies that important information might be lost. In comprehensive 2D chromatography, the entire sample is subjected to separation in both dimensions (Fanali *et al.*, 2013; Englert *et al.*, 2015). The drawback of comprehensive 2D chromatography is that this mode is much more difficult to perform in practice for on-line analysis and/or requires much longer analysis times and labour in the case of off-line analyses.

When the optimal 2D chromatographic conditions are sought, maximising the peak capacity is often the goal during method development (Kalili & De Villiers, 2013). Peak capacity refers to the maximum number of compounds resolvable by a particular chromatographic method (Giddings, 1967). The peak capacity is therefore a measure of the ability of a method to separate compounds, as a method with a higher peak capacity provides a better chance of separating a specific sample's components than a method with a lower peak capacity.

The peak capacity for HPLC separations in gradient mode can be determined using **equation 8** for 1D LC and **equation 9** for comprehensive 2D LC. **Equation 9** represents an approximation of the performance of comprehensive 2D LC, while **equation 10** can be used to account for the effect of first-dimension under-sampling (Jandera, 2012; Kalili & De Villiers, 2013). Note that values determined for comprehensive 2D LC according to equations 9 and 10 are only applicable if perfect orthogonality (see next section) is assumed.

$$n'_{c,1D} = 1 + \frac{t_g}{1/n\sum_1^n w_b} \quad (8)$$

$$n'_{c,2D} = {}^1n_c + {}^2n_c \quad (9)$$

$$n'_{c,2D} = \frac{{}^1n_c\,{}^2n_c}{\beta} = \frac{{}^1n_c\,{}^2n_c}{\sqrt{1+3.35({}^2t_c\,{}^1n_c/\,{}^1t_g)^2}} \quad (10)$$

Where:

$n'_{C,1D}$ = peak capacity of a 1D separation

t_g = gradient time

w_b = peak width at baseline

n = number of peaks

$n'_{C,2D}$ = (theoretical) peak capacity of a comprehensive 2D separation

^1n_c = peak capacity of the first dimension (^{1}D) separation

^2n_c = peak capacity of the ^{2}D separation

β = under-sampling correction factor

The discussion that follows will focus on comprehensive 2D chromatography only, as the technique was of importance for this study.

Comprehensive two-dimensional chromatography can be performed in either on-line or off-line modes. For on-line analyses, the two separations are directly connected, typically using a switching valve equipped with trapping loops. The analysis time of the ^{2}D should therefore be equal to the fraction collection time from the ^{1}D, which somewhat limits the performance of the ^{2}D (Horváth *et al.*, 2009). In addition to relatively short total analysis times, a further benefit of on-line 2D chromatography is that the sample is not exposed between the two analyses, minimising the risk of sample alteration/contamination. On the other hand, practical considerations make it difficult to achieve very high peak capacities. The mobile phase of the ^{1}D should be compatible with the stationary phase and detector of the ^{2}D as not to cause peak broadening or cause excessive noise.

In contrast, in off-line comprehensive 2D chromatography, the two separations are performed independently. Typically fractions from the ^{1}D are collected prior to their injection into the ^{2}D column. The advantage of this system is that the samples can be treated before injection into the ^{2}D, for example by evaporating the solvent and reconstitution in solvents that are compatible with the ^{2}D. The ^{2}D analysis time is also no longer dependent on the fraction collection time in off-line 2D chromatography. This implies that longer analysis times can be used in this dimension, providing overall higher performance. The disadvantages of off-line 2D chromatography is that it is labour intensive, loss of solutes and sample contamination can occur, it is difficult to automate and reproduce and is extremely time consuming. This mode of comprehensive 2D chromatography is normally used for highly complex samples or where instrumentation for on-line operation is not available (Qiu *et al.*, 2014; Liu *et al.*, 2015a).

Comprehensive 2D chromatographic data is normally presented in the form of contour plots. Raw data comprise a series of ^{2}D chromatograms, which are - placed next to each other in a matrix format, which can be visualised as a 2D contour plot or a 3D surf plot. An example of a contour plot obtained for the on-line comprehensive 2D LC analysis of rooibos tea phenolics is presented in **Figure 2.13** (Beelders *et al.*, 2012a).

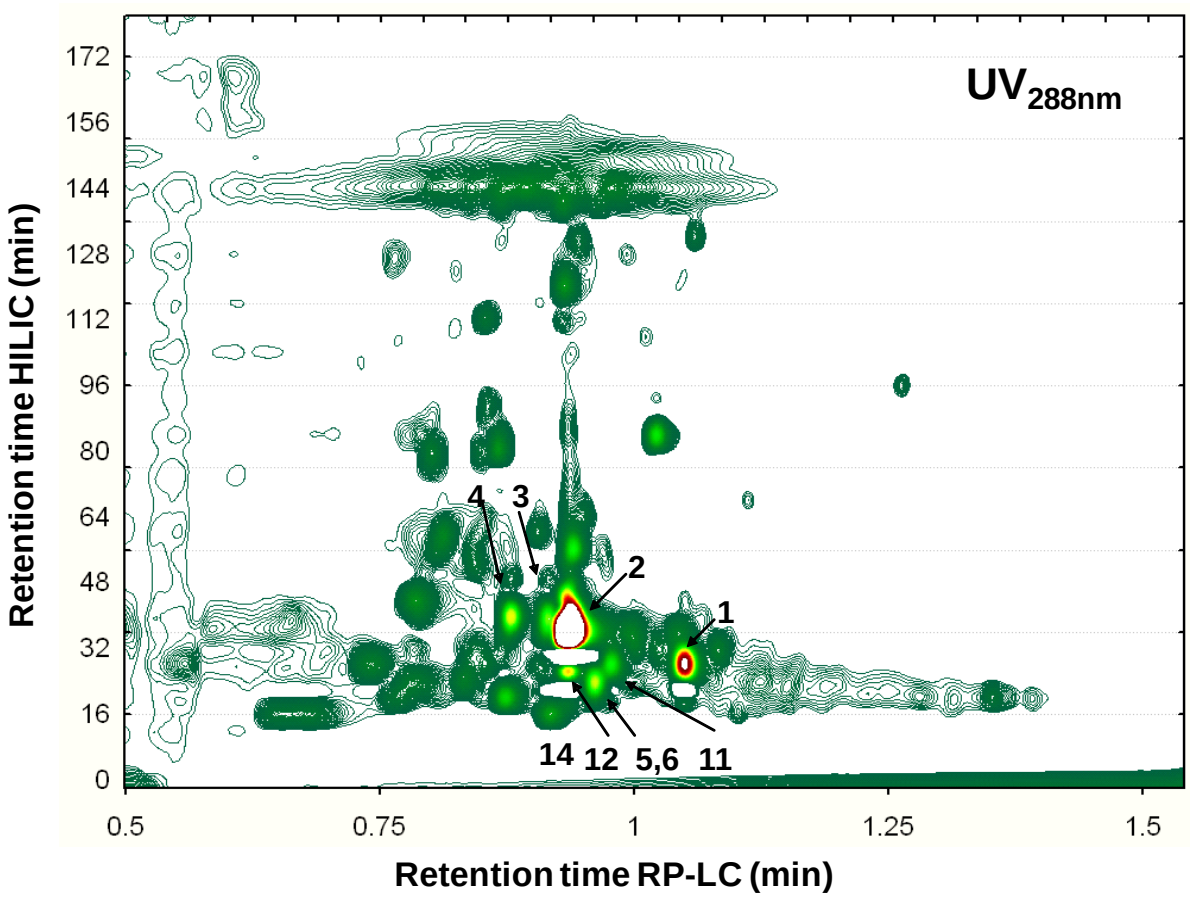


Figure 2.13 An example of a contour plot obtained for the (on-line) comprehensive two-dimension liquid chromatographic analysis of rooibos tea phenolics (Beelders *et al.*, 2012a). Note that the ^{1}D separation is presented on the y-axis in this figure.

5.2. Orthogonal Separation Mechanism and Implications

As alluded to above, it is required that the two separations combined in 2D chromatography are orthogonal if the benefits of the approach are to be exploited. For two separations to be completely orthogonal, the elution times of the two dimensions should be statistically independent; this can only be achieved if separation of compounds in the two dimensions are based on entirely different principles (Jandera, 2012). The degree of orthogonality determines the practically achievable resolving power. It is therefore important to take this parameter into account when estimating the performance of multidimensional separation techniques. **Equation 11** illustrates that a low orthogonality value (f_c) will reduce practical peak capacity, thereby lowering the potential of the comprehensive 2D separation. Several means of estimating f_c have been reported in literature (Semard *et al.*, 2010; Pourhaghighi *et al.*, 2011; Schure & Davis, 2015).

$$n'_{c,2D} = \frac{{}^{1}n_c \times {}^{2}n_c \times f_c}{\beta} \qquad (11)$$

Orthogonal metrics can be classified into two groups. Information theory is an example of a discretised metrics, while convex hull is classified as a non-discretised metrics. Discretisation here refers to the approach of dividing the measured space into ordered boxes of specific size and counting how many peaks are recorded within each of these boxes following the definition of the metric being used. To date no specific orthogonality metric system has been shown to be ideally suited to all comprehensive 2D data (Schure & Davis, 2015).

Methods that have been proposed to estimate orthogonality include those developed by Liu *et al.*, (1995) who used a geometric method. The parameters measured to obtain the degree of orthogonality were, peak spreading angle, retention correlation and practical peak capacity, from which a correlation matrix was derived and the degree of orthogonality determined. Gilar *et al.*, (2005) followed a different approach to determine orthogonality, as their method only used the retention times of separated compounds to determine the orthogonality. Another approach is the so-called convex hull method, which has been used with some success (Semard *et al.*, 2010; Schure & Davis, 2015). In this method, a convex hull is used to define how much of the available 2D separation space is occupied by/accessible to analyte peaks. The convex hull is calculated from the range-scaled retention times in two dimensions. A value between 0 and 1 is generated, where a value close to 1 characterises high orthogonality (Schure & Davis, 2015).

Pourhaghighi *et al.*, (2011) developed a novel method to determine the degree of orthogonality for 2D chromatography based on conditional entropy of information. Conditional entropy gives a measure of uncertainty for unknown/random variable(s) conditioned on known variable(s) (Shannon, 1948). Utilising conditional entropy to determine orthogonality for a 2D separation, the separation space, represented as a matrix, must be divided into spaces (boxes) of equal size, as the method requires discretised metrics. “Boxes” containing signal(s) are differentiated from “boxes” without signal(s). Using either the first or the second dimension compound retention times as the known variable, and the other dimension as the unknown variable (Wang *et al.*, 2009), the conditional entropy and orthogonality can be determined (Pourhaghighi *et al.*, 2011). The benefits of the method include that it measures the distribution of peaks across the separation space, and the methodology is practically independent of the number of chromatographic peaks.

As orthogonality is of high importance for 2D chromatography, the chosen modes of separation to be combined are critically important. In the case of comprehensive 2D LC, the combination of modes such as NP-LC, IEX, size exclusion chromatography (SEC) and RP-LC are powerful because the modes are based on different separation mechanisms (Li *et al.*, 2014). However, the modes to be used also depend on their compatibility with the sample components to be separated. From this perspective, the combination of CCC, as ^{1}D, and

HPLC, as ^{2}D, is promising, especially for off-line, comprehensive, 2D chromatography, because theoretically 100% of the sample compounds can be collected from a CCC column (Berthod *et al.*, 2009). The solvent system can be evaporated and the sample compounds reconstituted with a liquid matrix compatible with the HPLC column and samples. The robustness of CCC is highly beneficial when used as the ^{1}D. As a "new column" is used for each analysis, no column degradation should occur allowing near identical separation for each analysis (Berthod *et al.*, 2009). As HPLC have high efficiency and selectivity and is compatible with MS, ^{2}D analysis is ideal for HPLC analyses; UPHLC instruments can also be used to increase efficiency. A rapid (U)HPLC method can be designed to separate multiple compounds captured in each ^{1}D fraction.

5.3. Comprehensive 2D LC Separation of Phenolics

Comprehensive 2D LC separation methods have high resolving power (as reviewed by Francois *et al.*, 2009b), which lead to the increased popularity of using such techniques (Li *et al.*, 2014). As mentioned earlier in this chapter various LC modes have been combined for the purpose of comprehensive 2D LC. As polyphenolic compounds have similar polarities, comprehensive 2D LC methods are extremely useful as seen in Chen *et al.*, (2015). Off-line mode, although being more labour intensive, have a higher attainable practical peak capacity than on-line mode as the ^{2}D cycle time is not dependent on ^{1}D sampling time (Beelders *et al.*, 2012a), thereby allowing increased ^{2}D analysis time in which separation can be improved (see **equation 10**).

To date, only one study on the application of LC×LC to rooibos tea phenolics has been reported (Beelders *et al.*, 2012a). These authors reported a qualitative LC×LC method based on the combination of HILIC in the ^{1}D and RP-LC in the ^{2}D (**Figure 2.13**). Off-line and on-line HILIC×RP-LC analyses were compared in terms of peak capacity, analysis time and the advantages/disadvantages of each hyphenation mode. The off-line method provided better separation and orthogonality than the on-line system, but required much longer analysis times with a higher risk of sample degradation and contamination (Beelders *et al.*, 2012a).

5.4. Two-Dimensional Separations: Combining CCC and HPLC

The comprehensive combination of CCC and HPLC is particularly promising from the perspective of orthogonality, as these two techniques are based on different separation mechanisms. The comprehensive hyphenation of CCC and LC (CCC×LC) has for this reason also found application for the analysis of complex mixtures of phenolics in natural products.

Countercurrent chromatography performs exceptionally well as a preparative technique and for this reason it is well suited to be used as the ^{1}D where the compounds are initially separated and captured in fractions to be further analysed (Berthod, 2002a). The majority of CCC related separation methods use preparative or semi-preparative scale analysis, analytical scale separation are not commonly used. On the other hand, HPLC offers higher separation efficiency and has more parameters that influence separation, such as mobile phase composition, adsorption behaviour, ion exchange interactions and pore size exclusion. Therefore, if HPLC is used in the ^{2}D, the separation can be substantially improved and orthogonal separations may be achieved (Berthod, 2002a). This large number of parameters that affect selectivity in HPLC makes predicting the separation more difficult, but also provides more scope for improvement. RP-LC in particular is preferred as the ^{2}D separation as this mode can be directly coupled to DAD and MS detection and provides high chromatographic performance for relatively short analysis times (CCC can mostly not be directly coupled to MS due to the high flow rates and incompatible solvents).

Preparative off-line 2D CCC×HPLC-MS was used by Rodriguez-Rivera *et al.*, (2014) for metabolite profiling of polyphenols present in the peels of *Citrus limetta* Risso. This article is an excellent example of the preparative scale separation power of CCC in the ^{1}D, which allows high sample loading and sufficient analyte concentration per fraction when dried and reconstituted prior to HPLC analysis. The ^{2}D HPLC displayed much higher separation performance compared to CCC. The low flow rate of the HPLC analysis (0.25 ml.min^{-1}) with ESI-compatible mobile phases allowed for tentative identification of compounds without the need for an additional split valve to reduce the flow rate prior to MS detection.

Chen *et al.*, (2015) developed an on-line NP-CCC×RP-HPLC method to separate and isolate polar polyphenols present in Tieguanyin tea (Chinese oolong tea). The intriguing part of the research is how the authors coupled the ^{1}D to the ^{2}D as the solvents used for the ^{1}D (isopropanol and 16% sodium chloride aqueous solution (1:1.2, v/v)) was incompatible with the ^{2}D. First dimension fractions were directed via an electronically controlled switch to a holding column, where water was pump by a secondary preparative-HPLC pump to desalinate the fractions containing sample compounds. After desalination was completed, the trapped compounds were washed out in back-flush mode towards the ^{2}D for further separation.

Michel *et al.*, (2011) analysed *Hippophaë rhamnoides L*. berries using centrifugal partition chromatography (CPC) otherwise known as a hydrostatic CCC (**Figure 2.4A**) coupled in an on-line configuration to HPLC. The goal was to develop a separation method for flavonols using HPLC to guide the fraction collection parameters. As CPC is a low pressure technique and HPLC requires high pressure, the two methods cannot be directly

connected and the authors used a 6 port valve system, thereby eliminating the pressure difference hurdle. The MS used for tentative identification was not directly coupled to the HPLC separation (the final fractions were analysed using a separate HPLC-MS instrument), thereby removing the need for the method to be MS compatible.

References

Abad-Garcia, B., Berrueta, L. A., Garmon-Lobato, S., Gallo, B. & Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *Journal of Chromatography A,* **1216**, 5398-5415.

Abad-Garcia, B., Garmon-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2008). New features on the fragmentation and differentiation of *C*-glycosidic flavone isomers by positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry,* **22**, 1834-1842.

Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2009). Practical guidelines for characterization of *O*-diglycosyl flavonoid isomers by triple quadrupole MS and their applications for identification of some fruit juices flavonoids. *Journal of Mass Spectrometry,* **44**, 1017-1025.

Almajano, M. P., Carbó, R., Jiménez, J. A. L. & Gordon, M. H. (2008). Antioxidant and antimicrobial activities of tea infusions. *Food Chemistry,* **108**, 55-63.

Bączek, T. & Kaliszan, R. (2002). Combination of linear solvent strength model and quantitative structure–retention relationships as a comprehensive procedure of approximate prediction of retention in gradient liquid chromatography. *Journal of Chromatography A,* **962**, 41-55.

Baeza-Baeza, J. J., Davila, Y., Fernandez-Navarro, J. J. & Garcia-Alvarez-Coque, M. C. (2012). Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC. *Analytical and Bioanalytical Chemistry,* **404**, 2973-2984.

Beelders, T., Kalili, K. M., Joubert, E., De Beer, D. & De Villiers, A. (2012a). Comprehensive two-dimensional liquid chromatographic analysis of rooibos (*Aspalathus linearis*) phenolics. *Journal of Separation Science,* **35**, 1808-1820.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012b). Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation. *Journal of Chromatography A,* **1219**, 128-139.

Berthod, A. (2002a). Countercurrent Chromatography the Support-Free Liquid Stationary Phase. Pp. 397. Boston: Elsevier Science Ltd, Wilson & Wilson's Comprehensive Analytical Chemistry.

Berthod, A. (2002b). Separation of Inorganic Compounds: Theory, Methodology and Applications. In: Countercurrent chromatography the support-free liquid stationary phase (edited by Barceló, D.). Pp. 261-300. Amsterdam: Elsevier.

Berthod, A. (2007). Countercurrent chromatography and the journal of liquid chromatography: A love story. *Journal of Liquid Chromatography & Related Technologies,* **30**, 1447-1463.

Berthod, A., Hassoun, M. & Ruiz-Angel, M. J. (2005). Alkane effect in the Arizona liquid systems used in countercurrent chromatography. *Analytical and Bioanalytical Chemistry,* **383**, 327-340.

Berthod, A., Maryutina, T., Spivakov, B., Shpigun, O. & Sutherland, I. A. (2009). Countercurrent chromatography in analytical chemistry (IUPAC technical report). *Pure and Applied Chemistry,* **81**, 355-387.

Bottcher, S., Steinhauser, U. & Drusch, S. (2015). Off-flavour masking of secondary lipid oxidation products by pea dextrin. *Food Chemistry,* **169**, 492-498.

Brandt, K. & Mølgaard, J. P. (2001). Organic agriculture: does it enhance or reduce the nutritional value of plant foods? *Journal of the Science of Food and Agriculture,* **81**, 924-931.

Breiter, T., Laue, C., Kressel, G., Groll, S., Engelhardt, U. H. & Hahn, A. (2011). Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. *Food Chemistry,* **128**, 338-347.

Cabooter, D., Broeckhoven, K., Kalili, K. M., De Villiers, A. & Desmet, G. (2011). Fast method development of rooibos tea phenolics using a variable column length strategy. *Journal of Chromatography A,* **1218**, 7347-7357.

Cacciola, F., Donato, P., Giuffrida, D., Torre, G., Dugo, P. & Mondello, L. (2012). Ultra high pressure in the second dimension of a comprehensive two-dimensional liquid chromatographic system for carotenoid separation in red chili peppers. *Journal of Chromatography A,* **1255**, 244-251.

Cao, J., Yin, C., Qin, Y., Cheng, Z. & Chen, D. (2014). Approach to the study of flavone di-*C*-glycosides by high performance liquid chromatography-tandem ion trap mass spectrometry and its application to characterization of flavonoid composition in *Viola yedoensis*. *Journal of Mass Spectrometry,* **49**, 1010-1024.

Cavazzini, A., Pasti, L., Massi, A., Marchetti, N. & Dondi, F. (2011). Recent applications in chiral high performance liquid chromatography: a review. *Analytica chimica acta,* **706**, 205-222.

Charles, L. (2014). MALDI of synthetic polymers with labile end-groups. *Mass Spectrometry Review,* **33**, 523-543.

Chen, W. B., Li, S. Q., Chen, L. J., Fang, M. J., Chen, Q. C., Wu, Z., Wu, Y. L. & Qiu, Y. K. (2015). Online polar two phase countercurrent chromatography x high performance liquid chromatography for preparative isolation of polar polyphenols from tea extract in a single step. *Journal of Chromatography B,* **997**, 179-186.

Coetzee, G., Marx, I. J., Pengilly, M., Bushula, V. S., Joubert, E. & Bloom, M. (2008). Effectof rooibos and honeybush tea extracts against *Botrytis cinerea*. *South African Journal of Enology and Viticulture,* **9**, 33-38.

Costa, F., Garrard, I., Da Silva, A. J. & Leitão, G. G. (2013). Changes in the mobile phase composition on a stepwise counter-current chromatography elution for the isolation of flavonoids from *Siparuna glycycarpa*. *Journal of Separation Science,* **36**, 2253-2259.

Costa, F. & Leitao, G. G. (2011). Evaluation of different solvent systems for the isolation of *Sparattosperma leucanthum* flavonoids by counter-current chromatography. *Journal of Chromatography A,* **1218**, 6200-6205.

Costa, F. & Leitão, G. G. (2010). Strategies of solvent system selection for the isolation of flavonoids by countercurrent chromatography. *Journal of Separation Science,* **33**, 336-347.

Crozier, A., Jaganath, I. B. & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports,* **26**, 1001-1143.

Cullere, M., Hoffman, L. C. & Dalle Zotte, A. (2013). First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products. *Meat Science,* **95**, 72-77.

Cuyckens, F. & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry,* **39**, 1-15.

Cuyckens, F., Rozenberg, R., De Hoffmann, E. & Claeys, M. (2001). Structure characterization of flavonoid *O*-diglycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. *Journal of Mass Spectrometry,* **36**, 1203-1210.

De Beer, D., Malherbe, C. J., Beelders, T., Willenburg, E. L., Brand, D. J. & Joubert, E. (2015). Isolation of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) using high-performance countercurrent chromatography: Sample loading and compound stability considerations. *Journal of Chromatography A,* **1381**, 29-36.

De Villiers, A., Venter, P. & Pasch, H. (2016). Recent advances and trends in the liquid-chromatography-mass spectrometry analysis of flavonoids. *Journal of Chromatography A,* **1430**, 16-78.

Dludla, P. V., Muller, C. J., Louw, J., Joubert, E., Salie, R., Opoku, A. R. & Johnson, R. (2014). The cardioprotective effect of an aqueous extract of fermented rooibos (*Aspalathus linearis*) on cultured cardiomyocytes derived from diabetic rats. *Phytomedicine,* **21**, 595-601.

Drogue, S., Rolet, M. C., Thiébaut, D. & Rosset, R. (1991). Improvement of on-line detection in high-speed countercurrent chromatography: UV absorptiometry and evaporative light-scattering detection. *Journal of Chromatography A,* **538**, 91-97.

Dugo, P., Favoino, O., Luppino, R., Dugo, G. & Mondello, L. (2004). Comprehensive two-dimensional normal-phase (adsorption)−reversed-phase liquid chromatography. *Analytical Chemistry,* **76**, 2525-2530.

El-Aneed, A., Cohen, A. & Banoub, J. (2009). Mass spectrometry, review of the basics: electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews,* **44**, 210-230.

Englert, M., Brown, L. & Vetter, W. (2015). Heart-cut two-dimensional countercurrent chromatography with a single instrument. *Analytical Chemistry,* **87**, 10172-10177.

Faccin, H., Viana, C., Do Nascimento, P. C., Bohrer, D. & De Carvalho, L. M. (2016). Study of ion suppression for phenolic compounds in medicinal plant extracts using liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A,* **1427**, 111-124.

Fanali, C., Dugo, P., Mondello, L., D'Orazio, G. & Fanali, S. (2013). Recent developments in high performance liquid chromatography. In: *Food Analysis by HPLC* (edited by Nollet, L. M. L. & Toldra, F.). Pp. 24-25. New York: CRC Press.

Ferreres, F., Gil-Izquierdo, A., Andrade, P. B., Valentao, P. & Tomas-Barberan, F. A. (2007). Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A,* **1161**, 214-223.

Forcisi, S., Moritz, F., Kanawati, B., Tziotis, D., Lehmann, R. & Schmitt-Kopplin, P. (2013). Liquid chromatography-mass spectrometry in metabolomics research: mass analyzers in ultra high pressure liquid chromatography coupling. *Journal of Chromatography A,* **1292**, 51-65.

Foucault, A. P. & Chevolot, L. (1998). Counter-current chromatography: instrumentation, solvent selection and recent applications to natural product purification. *Journal of Chromatography A,* **808**, 3-22.

Francois, I., Cabooter, D., Sandra, K., Lynen, F., Desmet, G. & Sandra, P. (2009a). Tryptic digest analysis by comprehensive reversed phase x two reversed phase liquid chromatography (RP-LCx2RP-LC) at different pH's. *Journal of Separation Science,* **32**, 1137-1144.

Francois, I., Sandra, K. & Sandra, P. (2009b). Comprehensive liquid chromatography: fundamental aspects and practical considerations--a review. *Analytica chimica acta,* **641**, 14-31.

Friesen, B. & Pauli, G. F. (2005). G.U.E.S.S.—A generally useful estimate of solvent systems for CCC. *Journal of Liquid Chromatography & Related Technologies,* **28**, 2777-2806.

Friesen, J. B. & Pauli, G. F. (2007). Rational development of solvent system families in counter-current chromatography. *Journal of Chromatography A,* **1151**, 51-59.

Furey, A., Moriarty, M., Bane, V., Kinsella, B. & Lehane, M. (2013). Ion suppression; a critical review on causes, evaluation, prevention and applications. *Talanta,* **115**, 104-122.

Garrard, I. J., Janaway, L. & Fisher, D. (2007). Minimising solvent usage in high speed, high loading, and high resolution isocratic dynamic extraction. *Journal of Liquid Chromatography & Related Technologies,* **30**, 151-163.

Garrido Frenich, A., Plaza-Bolanos, P. & Martinez Vidal, J. L. (2008). Comparison of tandem-in-space and tandem-in-time mass spectrometry in gas chromatography determination of pesticides: application to simple and complex food samples. *Journal of Chromatography A,* **1203**, 229-238.

Ghiselli, A., Serafini, M., Maiani, G., Azzini, E. & Ferro-Luzzi, A. (1994). A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biology & Medicine,* **18**, 29-36.

Giddings, J. C. (1967). Maximum number of components resolvable by gel filtration and other elution chromatographic methods. *Analytical Chemistry,* **39**, 1027-1028.

Gilar, M., Olivova, P., Daly, A. E. & Gebler, J. C. (2005). Orthogonality of separation in two-dimensional liquid chromatography. *Analytical Chemistry,* **77**, 6426-6434.

González-Ruiz, V., Olives, A. I. & Martín, M. A. (2015). Core-shell particles lead the way to renewing high-performance liquid chromatography. *Trends in Analytical Chemistry,* **64**, 17-28.

Gouws, P., Hartel, T. & Van Wyk, R. (2014). The influence of processing on the microbial risk associated with rooibos (*Aspalathus linearis*) tea. *Journal of the Science of Food and Agriculture,* **94**, 3069-3078.

Gross, J. H. (2004). Mass Spectrometry A Textbook. Germany Heidelberg, Springer.

Hayes, R., Ahmed, A., Edge, T. & Zhang, H. (2014). Core-shell particles: preparation, fundamentals and applications in high performance liquid chromatography. *Journal of Chromatography A,* **1357**, 36-52.

Heim, K. E., Tagliaferro, A. R. & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of natural biochemistry,* **13**, 572-584.

Heinrich, T., Willenberg, I. & Glomb, M. A. (2012). Chemistry of color formation during rooibos fermentation. *Journal of Agricultural and Food Chemistry,* **60**, 5221-5228.

Hillis, W. E. & Inoue, T. (1967). The polyphenols of nothofagus species-II. The heartwood of *Nothofagus fusca*. *Phytochemistry,* **6**, 59-67.

Hoffman, L. C., Jones, M., Muller, N., Joubert, E. & Sadie, A. (2014). Lipid and protein stability and sensory evaluation of ostrich (*Struthio camelus*) droëwors with the addition of rooibos tea extract (*Aspalathus linearis*) as a natural antioxidant. *Meat Science,* **96**, 1289-1296.

Holcapek, M., Jirasko, R. & Lisa, M. (2012). Recent developments in liquid chromatography-mass spectrometry and related techniques. *Journal of Chromatography A,* **1259**, 3-15.

Horváth, K., Fairchild, J. N. & Guiochon, G. (2009). Generation and limitations of peak capacity in online two-dimensional liquid chromatography. *Analytical Chemistry,* **81**, 3879-3888.

Hübsch, Z., Van Vuuren, S. F. & Van Zyl, R. L. (2014). Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies? *South African Journal of Botany,* **93**, 148-156.

Ignat, I., Volf, I. & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry,* **126**, 1821-1835.

Ignatova, S., Sumner, N., Colclough, N. & Sutherland, I. (2011). Gradient elution in counter-current chromatography: a new layout for an old path. *Journal of Chromatography A,* **1218**, 6053-6060.

Iswaldi, I., Arraez-Roman, D., Rodriguez-Medina, I., Beltran-Debon, R., Joven, J., Segura-Carretero, A. & Fernandez-Gutierrez, A. (2011). Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Analytical and Bioanalytical Chemistry,* **400**, 3643-3654.

Jandera, P. (2012). Comprehensive two-dimensional liquid chromatography — practical impacts of theoretical considerations. A review. *Central European Journal of Chemistry,* **10**, 844-875.

Jerz, G., Wybraniec, S., Gebers, N. & Winterhalter, P. (2010). Target-guided separation of *Bougainvillea glabra* betacyanins by direct coupling of preparative ion-pair high-speed countercurrent chromatography and electrospray ionization mass-spectrometry. *Journal of Agricultural and Food Chemistry,* **1217**, 4544-4554.

Jiwan, J. L., Wallemacq, P. & Herent, M. F. (2011). HPLC-high resolution mass spectrometry in clinical laboratory? *Clinical Biochemistry,* **44**, 136-147.

Johnson, J. V., Yost, R. A., Kelley, P. E. & Bradford, D. C. (1990). Tandem-in-space and tandem-in-time mass spectrometry: triple quadrupoles and quadrupole ion traps. *Analytical Chemistry,* **62**, 2162-2172.

Jones, M., Hoffman, L. C. & Muller, M. (2015). Effect of rooibos extract (*Aspalathus linearis*) on lipid oxidation over time and the sensory analysis of blesbok (*Damaliscus pygargus phillipsi*) and springbok (*Antidorcas marsupialis*) droëwors. *Meat Science,* **103**, 54-60.

Joubert, E. (1996). HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chemistry,* **55**, 403-411.

Joubert, E., Beelders, T., De Beer, D., Malherbe, C. J., De Villiers, A. J. & Sigge, G. O. (2012). Variation in phenolic content and antioxidant activity of fermented rooibos herbal tea infusions: role of production season and quality grade. *Journal of Agricultural and Food Chemistry,* **60**, 9171-9179.

Joubert, E. & De Beer, D. (2011). Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany,* **77**, 869-886.

Joubert, E. & De Beer, D. (2014). Antioxidants of rooibos beverages: Role of plant composition and processing. In: Processing and Impact on Antioxidants in Beverages. Pp. 131-144.

Joubert, E., De Beer, D., Malherbe, C. J., Muller, N., Bonnet, S. L., Van der Westhuizen, J. H. & Ferreira, D. (2013). Occurrence and sensory perception of *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in rooibos (*Aspalathus linearis*). *Food Chemistry,* **136**, 1078-1085.

Joubert, E., Gelderblom, W. C. A., Louw, A. & De Beer, D. (2008). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Journal of Ethnopharmacology,* **119**, 376-412.

Joubert, E., Winterton, P., Britz, T. J. & Gelderblom, W. C. A. (2005). Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (*Aspalathus linearis*). *Journal of Agricultural and Food Chemistry,* **53**, 10260-10267.

Kalili, K. M. & De Villiers, A. (2013). Systematic optimisation and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography x reversed phase liquid chromatographic analysis of procyanidins, part I: Theoretical considerations. *Journal of Chromatography A,* **1289**, 58-68.

Kaliszan, R., Bączek, T., Bucinḱnski, A., Buszewski, B. & Sztupecka, M. (2003). Prediction of gradient retention from the linear solvent strength (LSS) model, quantitative structure-retention relationships (QSRR), and artificial neural networks (ANN). *Journal of Separation Science,* **26**, 271-282.

Kamakura, R., Son, M. J., De Beer, D., Joubert, E., Miura, Y. & Yagasaki, K. (2015). Antidiabetic effect of green rooibos (*Aspalathus linearis*) extract in cultured cells and type 2 diabetic model KK-A(y) mice. *Cytotechnology,* **67**, 699-710.

Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y. & Yagasaki, K. (2009). Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine,* **16**, 437-443.

Kazimierczak, R., Hallmann, E., Rusaczonek, A. & Rembiałkowska, E. (2013). Polyphenols, tannins and caffeine content and antioxidant activity of green teas coming from organic and non-organic production. *Renewable Agriculture and Food Systems,* **30**, 1-7.

Kebarle, P. (2000). A brief overview of the present status of the mechanisms involved in electrospray mass spectrometry. *Journal of Mass Spectrometry,* **35**, 804-817.

Koch, I. S., Muller, M., Joubert, E., Van der Rijst, M. & Næs, T. (2012). Sensory characterization of rooibos tea and the development of a rooibos sensory wheel and lexicon. *Food Research International,* **46**, 217-228.

Koch, I. S., Muller, N., De Beer, D., Næs, T. & Joubert, E. (2013). Impact of steam pasteurization on the sensory profile and phenolic composition of rooibos (*Aspalathus linearis*) herbal tea infusions. *Food Research International,* **53**, 704-712.

Koeppen, B. H. & Roux, D. G. (1965). Aspalathin: A novel *C*-glycosylflavanoid from *Aspalathus linearis*. *Tetrahedron Letters,* **39**, 3497-3503.

Kojima, H., Inagaki, M., Tomita, T., Watanabe, T. & Uchida, S. (2010). Improved separation and characterization of lipopolysaccharide related compounds by reverse phase ion pairing-HPLC/electrospray ionization-quadrupole-mass spectrometry (RPIP-HPLC/ESI-Q-MS). *Journal of Chromatography B,* **878**, 442-448.

Krafczyk, N. & Glomb, M. A. (2008). Characterization of phenolic compounds in rooibos tea. *Journal of Agricultural and Food Chemistry,* **56**, 3368-3379.

Krafczyk, N., Heinrich, T., Porzel, A. & Glomb, M. A. (2009). Oxidation of the dihydrochalcone aspalathin leads to dimerization. *Journal of Agricultural and Food Chemistry,* **57**, 6838-6843.

Kroon, P. A. & Williamson, G. (1999). Hydroxycinnamates in plants and food: current and future perspectives. *Journal of the Science of Food and Agriculture,* **79**, 355-361.

Ku, S. K., Kwak, S., Kim, Y. & Bae, J. S. (2015). Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibits high glucose-induced inflammation *in vitro* and *in vivo*. *Inflammation,* **38**, 445-455.

Kundu, R., Dasgupta, S., Biswas, A., Bhattacharya, S., Pal, B. C., Bhattacharya, S., Rao, P. G., Barua, N. C., Bordoloi, M. & Bhattacharya, S. (2011). Carlinoside reduces hepatic bilirubin accumulation by stimulating bilirubin-UGT activity through Nrf2 gene expression. *Biochemical Pharmacology,* **82**, 1186-1197.

Kwak, S., Han, M. S. & Bae, J. S. (2015). Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibit endothelial protein C receptor shedding *in vitro* and *in vivo*. *Fitoterapia,* **100**, 179-186.

Labuza, T. P. & Dugan, L. R. (1971). Kinetics of lipid oxidation in foods. *C R C Critical Reviews in Food Technology,* **2**, 355-405.

Lam, R. S. & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties using a structure-function approach. *Food Chemistry,* **141**, 975-984.

Li, D., Duck, R. & Schmitz, O. J. (2014). The advantage of mixed-mode separation in the first dimension of comprehensive two-dimensional liquid-chromatography. *Journal of Chromatography A,* **1358**, 128-135.

Lin, L. Z. & Harnly, J. M. (2012). Quantitation of flavanols, proanthocyanidins, isoflavones, flavanones, dihydrochalcones, stilbenes, benzoic acid derivatives using ultraviolet absorbance after identification by liquid chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry,* **60**, 5832-5840.

Liu, Q., Zeng, H., Jiang, S., Zhang, L., Yang, F., Chen, X. & Yang, H. (2015a). Separation of polyphenols from leaves of *Malus hupehensis* (Pamp.) Rehder by off-line two-dimensional high speed counter-current chromatography combined with recycling elution mode. *Food Chemistry,* **186**, 139-145.

Liu, Y., Friesen, J. B., McAlpine, J. B. & Pauli, G. F. (2015b). Solvent system selection strategies in countercurrent separation. *Planta Medica,* **81**, 1582-1591.

Liu, Y., Guo, Z., Feng, J., Xue, X., Zhang, F., Xu, Q. & Liang, X. (2009). Development of orthogonal two-dimensional hydrophilic interaction chromatography systems with the introduction of novel stationary phases. *Journal of Separation Science,* **32**, 2871-2876.

Liu, Z., Patterson Jr, D. G. & Lee, M. L. (1995). Geometric approach to factor analysis for the estimation of orthogonality and practical peak capacity in comprehensive two-dimensional separations. *Analytical Chemistry,* **67**, 3840-3845.

Malgas, R. R., Potts, A. J., Oettlé, N. M., Koelle, B., Todd, S. W., Verboom, G. A. & Hoffman, M. T. (2010). Distribution, quantitative morphological variation and preliminary molecular analysis of different growth forms of wild rooibos (*Aspalathus linearis*) in

the northern Cederberg and on the Bokkeveld Plateau. *South African Journal of Botany,* **76**, 72-81.

Mallet, C. R., Lu, Z. & Mazzeo, J. R. (2004). A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry,* **18**, 49-58.

Mamyrin, B. A. & Shmikk, D. V. (1979). The linear mass reflectron. *Soviet Physics—JETP,* **49**, 762-764.

Marais, C., Steenkamp, J. A. & Ferreira, D. (1996). Occurrence of phenylpyruvic acid in woody plants: biosynthetic significance and synthesis of an enolic glucoside derivative. *Journal of the Chemical Society, Perkin Transactions, 1,* **24**, 2915-2918.

March, R. E. (1997). An introduction to quadrupole ion trap mass spectrometry. *Journal of Mass Spectrometry,* **32**, 351-369.

Marnewick, J., Joubert, E., Joseph, S., Swanevelder, S., Swart, P. & Gelderblom, W. (2005). Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas. *Cancer Letters,* **224**, 193-202.

Marnewick, J. L., Rautenbach, F., Venter, I., Neethling, H., Blackhurst, D. M., Wolmarans, P. & Macharia, M. (2011). Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. *Journal of Ethnopharmacology,* **133**, 46-52.

Marnewick, J. L., Van der Westhuizen, F. H., Joubert, E., Swanevelder, S., Swart, P. & Gelderblom, W. C. (2009). Chemoprotective properties of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) herbal and green and black (*Camellia sinensis*) teas against cancer promotion induced by fumonisin B1 in rat liver. *Food and Chemical Toxicology,* **47**, 220-229.

Mazibuko, S. E., Muller, C. J., Joubert, E., De Beer, D., Johnson, R., Opoku, A. R. & Louw, J. (2013). Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (*Aspalathus linearis*). *Phytomedicine,* **20**, 813-819.

Michel, T., Destandau, E. & Elfakir, C. (2011). On-line hyphenation of centrifugal partition chromatography and high pressure liquid chromatography for the fractionation of flavonoids from *Hippophae rhamnoides L.* berries. *Journal of Chromatography A,* **1218**, 6173-6178.

Mondello, L., Donato, P., Cacciola, F., Fanali, C. & Dugo, P. (2010). RP-LC x RP-LC analysis of a tryptic digest using a combination of totally porous and partially porous stationary phases. *Journal of Separation Science,* **33**, 1454-1461.

Morris, H. R., Paxton, T. D. & Dell, A. (1996). High sensitivity collisionally-activated decomposition tandem mass spectrometry on a novel quadrupole/orthogonal-

acceleration time-of-flight mass spectrometer. *Rapid Communications in Mass Spectrometry,* **10**, 889-896.

Muller, C. J., Joubert, E., De Beer, D., Sanderson, M., Malherbe, C. J., Fey, S. J. & Louw, J. (2012). Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. *Phytomedicine,* **20**, 32-39.

Oh, J., Jo, H., Cho, A. R., Kim, S.-J. & Han, J. (2013). Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control,* **31**, 403-409.

Oka, H., Ikai, Y., Kawamura, N., Hayakawa, J., Harada, K. I., Murata, H., Suzuki, M. & Ito, Y. (1991). Direct interfacing of high-speed countercurrent chromatography to frit electron ionization, chemical ionization, and fast atom bombardment mass spectrometry. *Analytical Chemistry,* **63**, 2861-2865.

Oka, H. & Ito, Y. (1989). Improved method for continuous UV monitoring in high-speed counter-current chromatography. *Journal of Chromatography A,* **475**, 229-235.

Parejo, I., Jauregui, O., Sanchez-Rabaneda, F., Viladomat, F., Bastida, J. & Codina, C. (2004). Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. *Journal of Agricultural and Food Chemistry,* **52**, 3679-3687.

Pauli, G., Pro, S. M. & Friesen, B. (2008). Countercurrent separation of natural products. *Journal of natural products,* **71**, 1489-1508.

Pourhaghighi, M. R., Karzand, M. & Girault, H. H. (2011). Orthogonality of two-dimensional separations based on conditional entropy. *Analytical Chemistry,* **83**, 7676-7681.

Qiao, X., Ye, M., Liang, Y. H., Yang, W. Z. & Guo, D. A. (2011). Retention behaviors of natural products in reversed-phase liquid chromatography using mobile phase comprising methanol, acetonitrile and water. *Journal of Separation Science,* **34**, 169-175.

Qiu, Y. K., Yan, X., Fang, M. J., Chen, L., Wu, Z. & Zhao, Y. F. (2014). Two-dimensional countercurrent chromatography x high performance liquid chromatography for preparative isolation of toad venom. *Journal of Chromatography A,* **1331**, 80-89.

Remane, D., Meyer, M. R., Wissenbach, D. K. & Maurer, H. H. (2010). Ion suppression and enhancement effects of co-eluting analytes in multi-analyte approaches: systematic investigation using ultra-high-performance liquid chromatography/mass spectrometry with atmospheric-pressure chemical ionization or electrospray ionization. *Rapid Communications in Mass Spectrometry,* **24**, 3103-3108.

Rodriguez-Rivera, M. P., Lugo-Cervantes, E., Winterhalter, P. & Jerz, G. (2014). Metabolite profiling of polyphenols in peels of *Citrus limetta Risso* by combination of preparative

high-speed countercurrent chromatography and LC-ESI-MS/MS. *Food Chemistry,* **158**, 139-152.

Romanyshyn, L., Tiller, P. R., Alvaro, R., Pereira, A. & Hop, C. E. C. A. (2001). Ultra-fast gradient vs. fast isocratic chromatography in bioanalytical quantification by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry,* **15**, 313-319.

Sakushima, A., Nishibe, S., Takeda, T. & Ogihara, Y. (1988). Positive and negative ion mass spectra of flavonoid glycosides by fast atom bombardment. *Journal of the Mass Spectrometry Society of Japan,* **36**, 71-80.

Sasikiran Goud, E., Krishna Reddy, V. & Naresh Chandra Reddy, M. (2014). Development and validation of a reverse-phase liquid chromatographic method for determination of related substances of pitavastatin for 2 and 4 MG tablets. *International Journal of Pharmacy and Pharmaceutical Sciences,* **6**, 95-100.

Schure, M. R. & Davis, J. M. (2015). Orthogonal separations: Comparison of orthogonality metrics by statistical analysis. *Journal of Chromatography A,* **1414**, 60-76.

Semard, G., Peulon-Agasse, V., Bruchet, A., Bouillon, J. P. & Cardinael, P. (2010). Convex hull: a new method to determine the separation space used and to optimize operating conditions for comprehensive two-dimensional gas chromatography. *Journal of Chromatography A,* **1217**, 5449-5454.

Shannon, C. E. (1948). A mathematical theory of communication. *The Bell System Technical Journal,* **27**, 379-423, 623-656.

Skalicka-Wozniak, K. & Garrard, I. (2015). A comprehensive classification of solvent systems used for natural product purifications in countercurrent and centrifugal partition chromatography. *Natural Product Reports,* **32**, 1556-1561.

Snijman, P. W., Joubert, E., Ferreira, D., Li, X. C., Ding, Y., Green, I. R. & Gelderblom, W. C. (2009). Antioxidant activity of the dihydrochalcones aspalathin and nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) flavonoids, epigallocatechin gallate, and trolox. *Journal of Agricultural and Food Chemistry,* **57**, 6678-6684.

Snyder, L. R., Kirkland, J. J. & Glajch, J. L. (1997a). The column. In: Practical HPLC method development. Pp. 174-232. New York: Wiley.

Snyder, L. R., Kirkland, J. J. & Glajch, J. L. (1997b). Getting started. In: Practical HPLC Method Development. Pp. 1-20. New York, USA: John Wiley & Sons, Inc.

Steen, H., Kuster, B. & Mann, M. (2001). Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning. *Journal of Mass Spectrometry,* **36**, 782-790.

Sternson, L. A. (1983). Some strategies for improving specificity and sensitivity in the analysis of anti-cancer drugs. *Journal of Pharmaceutical and Biomecial Analysis,* **1**, 537-547.

Van der Merwe, J. D., Joubert, E., Manley, M., De Beer, D., Malherbe, C. J. & Gelderblom, W. C. (2010). *In vitro* hepatic biotransformation of aspalathin and nothofagin, dihydrochalcones of rooibos (*Aspalathus linearis*), and assessment of metabolite antioxidant activity. *Journal of Agricultural and Food Chemistry,* **58**, 2214-2220.

Villaño, D., Pecorari, M., Testa, M. F., Raguzzini, A., Stalmach, A., Crozier, A., Tubili, C. & Serafini, M. (2010). Unfermented and fermented rooibos teas (*Aspalathus linearis*) increase plasma total antioxidant capacity in healthy humans. *Food Chemistry,* **123**, 679-683.

Von Gadow, A., Joubert, E. & Hansmann, C. F. (1997). Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. *Journal of Agricultural and Food Chemistry,* **45**, 632-638.

Wang, Y., Huang, J. & Wang, H. (2009). A note on conditional entropy. In: Proceedings - 2009 2nd IEEE International Conference on Computer Science and Information Technology, ICCSIT 2009. Pp. 485-487.

Wollnik, H. (2015). High-resolving mass spectrographs and spectrometers. *Hyperfine Interactions,* **235**, 61-75.

Xu, T., Yang, M., Li, Y., Chen, X., Wang, Q., Deng, W., Pang, X., Yu, K., Jiang, B., Guan, S. & Guo, D. A. (2013). An integrated exact mass spectrometric strategy for comprehensive and rapid characterization of phenolic compounds in licorice. *Rapid Communications in Mass Spectrometry,* **27**, 2297-309.

Zhang, C. X., Sun, Z. P. & Ling, D. K. (1993). Micellar electrokinetic capillary chromatography theory based on conventional chromatography. *Journal of Chromatography A,* **655**, 309-316.

Chapter 3

Improved HPLC method for rooibos phenolics targeting changes due to fermentation

The chapter was accepted by the Journal of Food Composition and Analysis (co-authors: A. de Villiers, E. Joubert, D. de Beer).

Abstract

The phenolic composition of rooibos (*Aspalathus linearis*) herbal tea is inherently linked to its health-promoting properties. “Fermentation” (oxidation) develops the characteristic flavour and colour of the herbal tea product. Changes to the phenolic composition during fermentation include conversion of aspalathin, a dihydrochalcone unique to rooibos and the major bioactive compound, to eriodictyol-glucopyranoside isomers. These compounds have not been quantified in rooibos extracts to date, due to separation challenges. In this study an improved HPLC method using a core shell column was developed to provide effective separation of 16 rooibos tea phenolics (aspalathin, four eriodictyol-glucopyranoside isomers and 12 other major phenolic compounds) in a reasonable time. Compounds were tentatively identified using authentic reference standards and/or tandem mass spectrometry. The HPLC method utilising diode-array detection for quantitation was validated. After evaluation of different extraction solvents, a 40% aqueous acetonitrile solution was deemed optimal for extraction of phenolic compounds from rooibos plant material. Green, semi-fermented and fermented plant material from 10 sub-divided rooibos bushes were extracted and analysed. Water extracts, representing food ingredient extracts, were also analysed. The largest decreases with fermentation were observed for the dihydrochalcones, with moderate to small decreases for flavonols. The levels of the eriodictyol-glucopyranoside isomers increased following fermentation.

1. Introduction

Rooibos (*Aspalathus linearis* (Burm.f.) R.Dahlgren) is an endemic South African plant that grows naturally in the fynbos biome in the Cederberg area (Malgas *et al.*, 2010). Rooibos is mostly cultivated for the purpose of herbal tea production; rooibos is processed to obtain a sought-after herbal tea that is extensively sold in South Africa. Major international markets are Germany, the Netherlands, the United Kingdom, the United States of America and Japan. The discovery in 1968 that administration of rooibos tea to an infant suffering from various allergic reactions helped reduce colic, and the more recent advent of natural antioxidants as "lifespan essentials", has led to further investigation of the beneficial health effects of rooibos products (Joubert & De Beer, 2011).

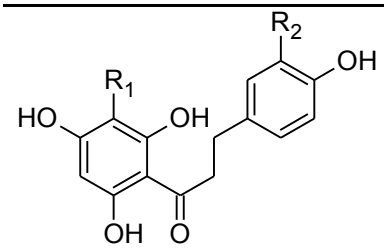
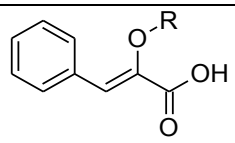
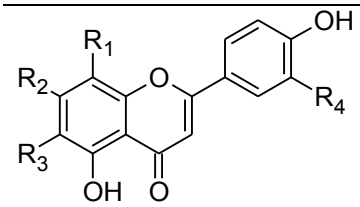
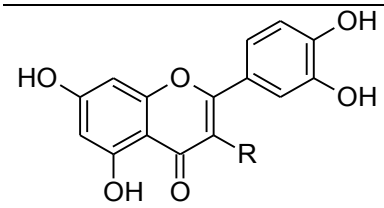
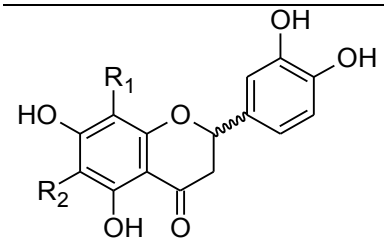
A major focus of the beneficial effects of rooibos and, in particular, its major flavonoid, the dihydrochalcone aspalathin, has been on its anti-diabetic properties (Muller *et al.*, 2016). Aspalathin is exclusively found in rooibos plant material (Joubert *et al.*, 2008; De Beer *et al.*, 2016). Most of the herbal tea is consumed in "fermented" form. "Fermentation" of the plant material induces oxidation of the phenolic compounds as a result of cutting, bruising and exposure to air. During this process, the aspalathin content rapidly declines, with little aspalathin remaining at the end of fermentation (Joubert, 1996). Oxidation of aspalathin results in the formation of eriodictyol-glucopyranoside flavanone isomers as intermediate products (**Table 3.1**), with further oxidation of the flavanones producing the flavones iso-orientin and orientin (Krafczyk & Glomb, 2008). Aspalathin and its oxidation products may be further oxidised to form high molecular weight brown compounds via polymerisation reactions (Heinrich *et al.*, 2012), thus contributing to the characteristic red-brown colour of rooibos. Other phenolic compounds present in unfermented rooibos, such as nothofagin, may also undergo enzymatic and/or chemical degradation, resulting in various phenolic reaction products.

In view of the contribution of the phenolic composition of rooibos tea in determining the properties of the product, accurate quantitative analysis of these compounds is of clear importance. To date, the most comprehensive analysis of rooibos phenolics has been achieved by the quantitative method of Beelders *et al.*, (2012). However, the method was largely developed to quantify the major phenolic compounds in fermented rooibos, therefore little attention was given to the intermediate oxidation products of aspalathin, namely (*R*)- and (*S*)-eriodictyol-6-*C*-glucopyranoside and -8-*C*-glucopyranoside. Another important consideration in the quantitative analysis of the phenolic content of plant material is the extraction solvent. Water extracts are mostly used to reflect the content of a cup of tea (Joubert & De Beer, 2012), although organic solvents such as acetonitrile or ethanol may

also be used to improve the extraction efficiency of phenolic compounds (Pasrija & Anandharamakrishnan, 2015).

The objective of this study was therefore to develop and validate a reversed phase (RP) HPLC method using diode-array detection (DAD) on conventional HPLC instrumentation, specifically targeting the flavanone intermediate oxidation products of aspalathin. The method developed by Beelders *et al.*, (2012) was used as a starting point for method development. Validation included determining linearity and repeatability, as well as the specificity of the method using high resolution (tandem) MS to confirm peak purity and identity. Extraction of phenolic compounds from rooibos plant material was optimised using mixtures of water, ethanol and acetonitrile. The RP-HPLC method was then applied to determine the effect of fermentation on the phenolic composition of rooibos plant material (using the optimised extraction solvent). Simulated industrial food ingredient extracts prepared from each of the rooibos samples were analysed to quantify the differences in their phenolic composition as a result of fermentation.

Table 3.1 Structures of the phenolic analytes of interest for HPLC method

Structure	Compound	Substituents
	**Dihydrochalcones**	
	Aspalathin	R_1 = β-D-glucopyranosyl, R_2 = OH
	Nothofagin	R_1 = β-D-glucopyranosyl, R_2 = H
	**Phenylpropanoid**	
	Phenylpyruvic acid-2-*O*-glucopyranoside (PPAG)	R = glucopyranosyl
	**Flavones**	
	Iso-orientin	R_1 = H, R_2 = R_4 = OH, R_3 = β-D-glucopyranosyl
	Orientin	R_1 = β-D-glucopyranosyl, R_2 = R_4 = OH, R_3 = H
	Vitexin	R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = R_4 = H
	Isovitexin	R_1 = R_4 =H, R_2 = OH, R_3 = β-D-glucopyranosyl
	Luteolin-7-*O*-glucopyranoside	R_1 = R_3 = H, R_2 = β-D-glucopyranosyloxy, R_4 = OH
	**Flavonols**	
	Isoquercitrin	R = β-D-glucopyranosyloxy
	Hyperoside	R = β-D-galactopyranosyloxy
	Quercetin-3-*O*-robinobioside	R= α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyloxy
	Rutin	R = α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy
	**Flavanones**	
	(*R*)/(*S*)-eriodictyol-8-*C*-glucopyranoside	R_1 = β-D-glucopyranosyl, R_2 = H
	(*R*)/(*S*)-eriodictyol-6-*C*-glucopyranoside	R_1 = H, R_2 = β-D-glucopyranosyl

2. Materials and Methods

2.1. Chemicals

HPLC grade water was prepared by purifying deionised water using Millipore Elix and Milli-Q academic (Millipore, Bedford, USA) water purification systems in series. HPLC gradient grade acetonitrile for HPLC-DAD analysis was purchased from Merck (Darmstadt, Germany), super purity acetonitrile (far UV) solvent for LC-MS analyses from Romil (Cambridge, United Kingdom) and glacial acetic acid from Sigma-Aldrich (St Louis, MO, USA). Authentic reference standards (purity ≥ 95%) were purchased from Extrasynthese (Genay, France; iso-orientin, orientin, eriodictyol-7-*O*-glucopyranoside, isovitexin, hyperoside), Karl Roth (Karlsruhe, Germany; vitexin, luteolin-7-*O*-glucopyranoside), Sigma-Aldrich (isoquercitrin), Transmit (Gießen, Germany; rutin) and the PROMEC unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa; aspalathin and nothofagin). Enolic phenylpyruvic acid-2-*O*-glucopyranoside (PPAG) was from the compound library of the Post-Harvest & Wine Technology Division of the Agricultural Research Council (Infruitec-Nietvoorbij) of South Africa. The phenolic standard stock solutions were prepared in dimethyl sulphoxide (DMSO) at concentrations of approximately 1 $mg.mL^{-1}$ and diluted with water as required. All the diluted standard mixtures contained ca. 1% ascorbic acid ($m.v^{-1}$) (Sigma-Aldrich) and were filtered through 0.22 µm hydrophilic PVDF filters (Millipore) before use.

2.2. Preparation of Rooibos Plant Material

Shoots (leaves and stems) were harvested during summer from ten bushes randomly selected from a commercial plantation on Nooitgedacht farm (Clanwilliam, South Africa) and the shoots from each plant were kept separate. Immediately prior to processing, the top and bottom 5 cm of the shoots were removed.

Green (unfermented) rooibos was prepared by drying ca. one-third of the “intact” shoots of a sample without delay at 40 °C for 12 h in a cross-flow drying tunnel. The remaining shoots were shredded into small pieces (< 3 mm) and mixed thoroughly. A sub-sample of the shredded plant material was dried 1 h after shredding, representing semi-fermented rooibos. For preparation of fermented rooibos, 300 g shredded plant material was moistened with 130 mL water, incubated at 38 °C for 12 h and then dried as described above. The dried plant material was milled using a Retsch mill (1 mm sieve; Retsch GmbH, Haan, Germany).

2.3. Preparation of Rooibos Water Extracts

All the rooibos samples underwent the same hot water extraction process as described by Joubert & De Beer, (2012). Briefly, 40 g of milled plant material was extracted with freshly-boiled deionised water (400 mL). Extraction was performed in a pre-heated waterbath at 93 °C for 30 min in a closed laboratory bottle while the extraction mixture was agitated every 5 min. After 30 min, the warm mixture was filtered through Whatman #4 filter paper. The filtrates were freeze-dried and stored in sealed vials in a desiccator below 5 °C. The extracts were reconstituted in water to a concentration of ca. 2.3 $mg.mL^{-1}$ and aliquots were frozen until analysis. Before analysis, ascorbic acid was added to the defrosted samples at ca. 1% final concentration and filtered using 0.22 µm hydrophilic PVDF syringe filters (Millipore).

2.4. Optimisation of Plant Material Extraction

One of each of the fermented, semi-fermented and green, milled rooibos plant material samples originating from the same bush was used to determine the optimal extraction solvent. Different ethanol-water and acetonitrile-water mixtures were evaluated, namely 0, 20, 40, 60, 80 and 100% ($v.v^{-1}$). Prior to extraction the milled plant material was further refined using a Retsch MM301 ball mill to improve sample homogeneity for mini-scale extraction. The finely milled rooibos plant material samples were subjected to extraction as described by Joubert *et al.*, (2014). Briefly, 3 mL solvent was added to ca. 80 mg of plant material in a 4 mL reaction vial, followed by heating in a digitally-controlled heating block at 100 °C for 20 min. Upon removal from the heating block, the samples were sonicated for 5 min and cooled to room temperature. The extract was filtered using a 0.45 µm hydrophilic PVDF syringe filter (Millipore), diluted and ascorbic acid solution (final concentration 0.1-0.2%) added. All treatments were repeated in triplicate and aliquots were frozen until analysis.

After the optimal extraction solvent was selected (40% aqueous acetonitrile), the fermented (n = 10), semi-fermented (n = 10), and green (n = 10) plant material was extracted with 40% aqueous acetonitrile using the procedure described above. Duplicate extracts were prepared and aliquots were frozen until analysis.

2.5. Instrumentation

An Agilent HPLC 1200 series instrument (maximum pressure limit 400 bar) equipped with an in-line degasser, quaternary pump, autosampler, column thermostat with an in-line 3 µL sample preheater and a diode array detector (DAD), was used for method development and quantification of extracts. The instrument was controlled using Chemstation software (Agilent Technologies, Waldbronn, Germany). All HPLC analyses were performed using an organic phase consisting of acetonitrile (B) and an aqueous phase consisting of 2% aqueous acetic acid ($v.v^{-1}$) (A).

LC-MS analyses were conducted on a UPLC instrument coupled to a Synapt G2 quadrupole-time-of-flight (Q-TOF) MS detector equipped with an electrospray ionisation (ESI) source (Waters, Milford, USA). A maximum pressure of 600 bar was used as this is the pressure limit of the Poroshell column. The UPLC instrument was equipped with a binary solvent manager, sample manager, column heating compartment, pre-column heater and a DAD detector, controlled using MassLynx software (Waters). The detector was equipped with a 500 nL flow cell.

2.6. Column Performance Evaluation

Performance evaluation of ZORBAX SB C_{18} (100 x 4.6 mm i.d. 1.8 µm; Agilent Technologies) columns, previously used by Beelders *et al.*, (2012) for rooibos phenolics, was conducted using isocratic elution with a mobile phase consisting of 65% acetonitrile and 35% water. The column temperature was set to 23 °C and the flow rate was 1.4 $mL.min^{-1}$. The performance of Poroshell 120 columns (100 x 4.6 mm and 150 x 4.6 mm i.d. both 2.7 µm; Agilent Technologies) was evaluated using isocratic elution with 70% acetonitrile and 30% water as mobile phase at a flow rate of 1.8 $mL.min^{-1}$ and column temperature of 23 °C.

For both column types the same standard mixture (3 µL; ALO 3045, Phenomenex, Torrance, USA), containing uracil, acetophenone, toluene and naphthalene, was injected in triplicate. The number of theoretical plates of each column was calculated for naphthalene using the half-height method and the Chemstation software, and compared to the value reported by the column manufacturer.

2.7. Chromatographic Conditions

2.7.1. HPLC-DAD Method Development

In this study, three ZORBAX C_{18} (100 x 4.6 mm i.d. 1.8 µm) columns, each from a different batch, and two Agilent Poroshell 120 C_{18} columns of different lengths (100 x 4.6 mm i.d. and 150 x 4.6 mm i.d. both with 2.7 µm particles) were evaluated.

The method described by Beelders *et al.*, (2012) was used as starting point for method development, with the aim of improving the separation of eriodictyol-glucopyranoside stereo-isomers (**Table 3.1**).

Initially the starting organic mobile phase concentration was changed in intervals of 1% per trial, ranging between 5 and 12%. The change in the overall mobile phase gradient steepness was compensated for by adapting the method accordingly. The method was also tested without compensating for the overall change in the mobile phase gradient. The best separation was obtained at a starting organic mobile phase concentration of 10%.

The second parameter evaluated, was the isocratic holding period (IHP). The IHP was removed, halved and doubled. Removing the IHP resulted in the most favourable separation and was used in further method development.

The third parameter evaluated, was column temperature; ranges of 28-48 °C for the ZORBAX columns and 25-48 °C for the Poroshell columns were assessed at intervals of 2 °C or less. The best separation on the ZORBAX C_{18} column remained at 37 °C as used by Beelders *et al.*, (2012) whereas for the Poroshell 120 columns it was 44.5 °C.

Finally, a 150 x 4.6 mm i.d. 2.7 µm Poroshell 120 column was selected (as opposed to the 100 mm column used in method development), as this improved separation efficiency.

2.7.2. Quantitative HPLC-DAD Analysis and Method Validation

A Poroshell 120 (150 x 4.6 mm i.d. 2.7 µm) column, protected by a ZORBAX SB C_{18} analytical guard column (12.5 x 4.6 mm i.d. 5 µm) (Agilent) and a Waters Acquity in-line filter, was used to analyse extracts in duplicate. The flow rate was 1 $mL.min^{-1}$ and a multilinear gradient was used as follows: 10-14.8% B (0-28.5 min), 14.8-19.2% B (28.5-33 min), 19.2-100% B (33-33.5 min), 100% B isocratic (33.5-38 min), 100-10% B (38-39 min), 10% B isocratic (39-46 min). The column oven temperature was set to 44.5 °C. UV spectra were recorded between 200 and 700 nm with selected wavelength monitoring at 288 nm and 350 nm.

Method validation included evaluation of linearity, intra-day repeatability (6 repeat injections), as well as sample and standard stability (24 h) (Shabir *et al.*, 2007). The method was used in conjunction with high resolution MS to confirm the identity and purity of quantified peaks. The flavanones, dihydrochalcones and PPAG were quantified at 288 nm, whilst the flavones and flavonols were quantified at 350 nm. The flavanones were quantified as eriodictyol-7-*O*-glucopyranoside equivalents, as no authentic reference standards were available. For the same reason, quercetin-3-*O*-robinobioside was quantified in terms of rutin equivalents. Nothofagin and PPAG were quantified using response factors relative to aspalathin: namely 1.346 and 1.016, respectively. The calibration mixtures were injected at eight different injection volumes, ranging from 0.009-2.6 µg injected on-column. This range was chosen to cover the expected concentration range in samples. Linear regression was performed for each compound to determine the slope, y-intercept and correlation coefficient (r^2) using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

2.7.3. Liquid Chromatography (LC)-ESI-MS and LC-ESI-MS/MS Analyses

LC-MS analyses were performed using the optimised HPLC method**,** except that the in-line filter and guard column were removed due to size constraints in the UPLC column oven.

This resulted in a small selectivity change, which was corrected for by raising the column temperature to 47 °C.

The mass spectrometer was operated in positive and negative ESI modes. The eluent was split in a 7:3 ratio before entering the ionisation chamber. Mass calibration was performed using a sodium formate solution and leucine encephalin was used as the lockspray solution. A mass range of 150-1500 amu was scanned and MassLynx v.4.1 (Waters) software was used to process the data. The capillary voltage was 2.5 and -2.5 kV for positive and negative ionisation, respectively, and the sampling cone voltage was set to 15.0 V. The source and desolvation temperatures were 120 °C and 275 °C, respectively. The desolvation and cone gas flows (N_2) were 650 and 50 $L.hr^{-1}$, respectively. For the MS/MS experiments, a collision energy of 30.0 V was used.

2.8. Statistical Analysis

Quantitative data were subjected to analysis of variance (ANOVA) using SAS® version 9.2 software (SAS Institute, Cary, NC, USA). The Shapiro-Wilk test was performed to test for normality and remove outliers. The student t-test was used to calculate the least significant difference (LSD) at a 5% level. Values of $p < 0.05$ were considered to be significant. A principal component analysis (PCA) was performed using mean centered data obtained from the extraction of the plant material with different solvents to visualise the optimal extraction solvent for overall extraction of rooibos phenolics. The student t-test and PCA were performed using XLstat software (version 2014, Addinsoft, New York, USA).

3. Results and Discussion

3.1. High Performance Liquid Chromatography (HPLC)-DAD Method Development

The RP-HPLC method of Beelders *et al.*, (2012) was not suitable for the quantification of the eriodictyol-glucopyranosides and other early eluting compounds of rooibos as they were not completely separated. The goal was therefore to improve the separation of the early eluting compounds, while still retaining the separation of the later eluting compounds in an acceptable separation time. However, analyses using the method reported by Beelders *et al.*, (2012) on a new ZORBAX SB C_{18} column failed to provide the same separation (**Figure 3.1A**). Separation of two critical peak pairs, namely iso-orientin and orientin, as well as quercetin-3-*O*-robinobioside and vitexin, was compromised (**Figure 3.1B**). This prompted an investigation into the column performance of three new columns as well as the original column used in Beelders *et al.*, (2012) (all columns have the same product code, but are from different production batches). Column performance was evaluated by measuring the number of theoretical plates (N) for naphthalene according to the manufacturer's criteria

(**Table 3.2**). In addition, retention times for the critical peak pairs were compared to those of the reference method (Beelders *et al.*, 2012) under identical conditions.

Table 3.2 Retention times (t_R) and resolution (R_s) for two critical peak pairs determined using gradient analysis according the method of Beelders *et al.*, (2012) on several ZORBAX SB C_{18} columns. Plate numbers (N) for naphthalene were determined using isocratic analyses

Column[a]	Batch	t_R (min)				R_s		N[c]
		Peak a[b]	Peak b[b]	Peak c[b]	Peak d[b]	Peak a and b[b]	Peak c and d[b]	
A	1	9.94	10.45	13.80	14.10	2.15	1.05	16140
B	2	9.58	9.86	13.29	13.29	1.09	-[d]	17017
C	3	9.87	10.07	13.60	13.60	0.62	-[d]	15436
D	4	9.86	10.15	13.62	13.62	1.14	-[d]	15467

[a]Columns were all ZORBAX SB C_{18} (100 x 4.6 mm i.d. 1.8 µm) from the same supplier. Batch numbers, 1 = USWFM02081; 2 = USWFM02350; 3 = USWFM02346; 4 = USWFM02515.

[b]Peak labels as defined in **Figure 3.1**.

[c]Number of theoretical plates determined by isocratic elution at 65% acetonitrile/35% water calculated using the half height method by the Chemstation software (n = 3).

[d]Complete co-elution of compounds.

Column A, used by Beelders *et al.*, (2012), gave good separation of the major rooibos phenolic compounds, including the critical peak pairs (**Table 3.2**). However, this column had been used extensively (> 2200 injections) and could be close to the end of its lifetime. Columns B, C and D were all new columns purchased in early 2015. None of these columns provided separation of quercetin-3-*O*-robinobioside and vitexin under the same conditions as used for column A, while the separation of iso-orientin and orientin was also worse than observed for column A. The column efficiencies were roughly comparable, but not their selectivities (**Table 3.2**). The observed differences in selectivity might be caused by any of a number of factors, such as differences in the derivatisation/deactivation reactions used during manufacturing or batch-to-batch variation in the silica particles used (Snyder *et al.*, 1997); insufficient information is available to accurately assess the cause of the observed differences. Changing the temperature, mobile phase gradient and IHP, as well as mobile phase flow rate proved unsuccessful to obtain the same separation observed for column A (**Figure 3.1A**) on any of the newer columns. The use of a longer column was not an option, due to pressure limitations of the equipment and column. These small but significant changes in selectivity therefore imply that the previous method was not sufficiently robust for routine analysis of rooibos phenolics in light of the observed batch-to-batch variations observed for the ZORBAX SB C_{18} columns.

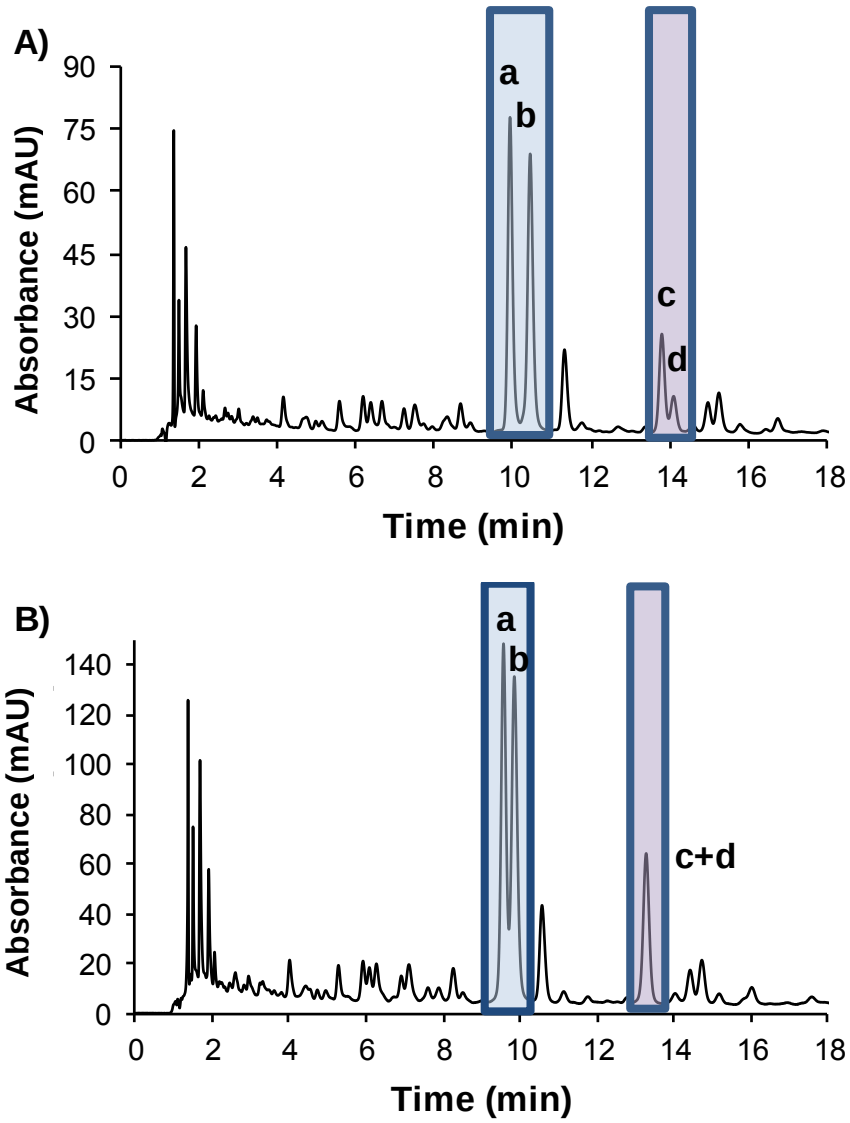


Figure 3.1 Chromatograms (350 nm) obtained for the gradient analysis of a fermented rooibos extract using the method of Beelders *et al.*, (2012) on two different ZORBAX SB C_{18} columns indicating selectivity differences between columns of different batch numbers: (A) column A and (B) column B (for column details refer to **Table 3.2**). **a** = iso-orientin, **b** = orientin, **c** = quercetin-3-*O*-robinobioside, **d** = vitexin.

For the development of an alternative HPLC method, a Poroshell 120 C_{18} column (100 x 4.6 mm i.d. 2.7 µm) was selected, as this column displays similar selectivity to the ZORBAX SB C_{18} column. Furthermore, the core-shell stationary phase used in the Poroshell column offers the benefit of similar performance to fully porous phases of smaller particle sizes at lower pressures, thereby allowing the use of longer columns on conventional HPLC instrumentation (Hayes *et al.*, 2014; González-Ruiz *et al.*, 2015).

Identical conditions as those used in the original method (Beelders *et al.*, 2012) were used as starting point for method development on the Poroshell column. As a first step,

different initial organic mobile phase ratios and gradient steepness levels were evaluated. These were left unchanged from the original method, as any changes resulted in a loss of overall separation, especially for orientin and iso-orientin. Changes in the IHP were also evaluated: the IHP was entirely removed as this resulted in better separation of the major phenolic compounds and the four early eluting eriodictyol-glucopyranosides. The effect of column temperature was evaluated as well. In contrast to the ZORBAX SB C_{18} columns, where a temperature of 37 °C was optimal, a temperature of 44.5 °C provided the best separation of early and late eluting compounds on the Poroshell 120 columns. Finally, in order to improve separation of some critical peak pairs, a longer column (150 mm vs. 100 mm) was selected. Note that this column could still be used on a system with a maximum pressure of 400 bar under optimal conditions due to the higher permeability of the core-shell phase (Hayes *et al.*, 2014; González-Ruiz *et al.*, 2015).

The final method on the Poroshell column provided satisfactory separation of all the principal rooibos tea phenolics, including the eriodictyol-glucopyranosides **1**-**4** (**Figure 3.2**). The new method does suffer from some disadvantages, primarily the fact that the separation of ferulic acid and aspalathin was lost. This will, however, not affect the quantitative data for aspalathin significantly, as ferulic acid is present at much lower levels than aspalathin in rooibos water extracts (Beelders *et al.*, 2012) and also displays relatively low absorbance at the quantification wavelength used for aspalathin (288 nm). Due to the improved separation achieved for the eriodictyol-glucopyranosides (compound **1**-**4**), which was the aim of the current study, this separation trade-off was deemed acceptable.

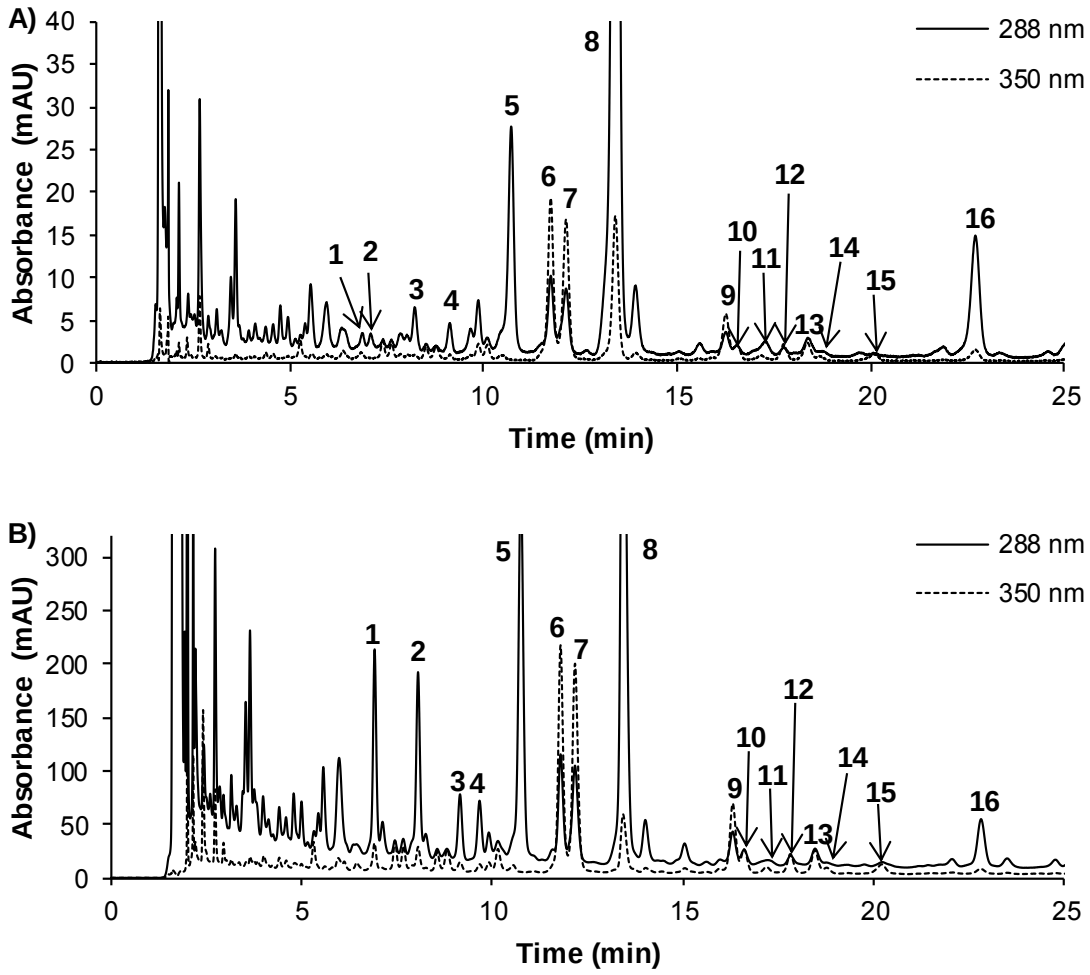


Figure 3.2 RP-HPLC-DAD chromatograms for green (A) and fermented (B) rooibos water extracts obtained using the developed method on a Poroshell 120 column. For experimental conditions, refer to Section 2.7.2. Peak numbers correspond to numbers in **Table 3.3**.

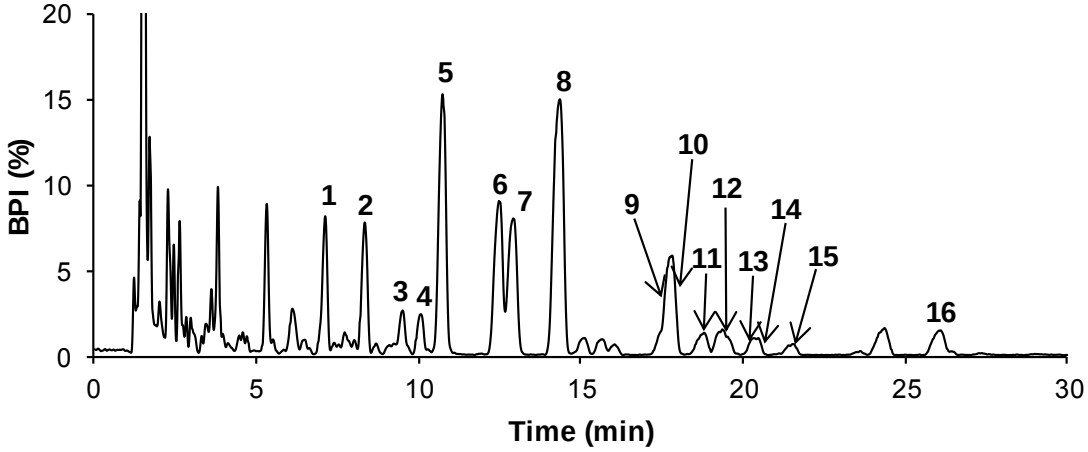


Figure 3.3 Base peak chromatogram (negative ionisation mode) of a fermented rooibos water extract. The peak numbers correspond to numbers in **Table 3.3**.

Table 3.3 UV-Vis and MS characteristics of polyphenolic compounds quantified in rooibos

t_R (min)	Nr	Negative ionisation	Positive ionisation						
		Accurate mass exp $[M-H]^-$	Accurate mass exp $[M+H]^+$	Accurate mass calc	Proposed molecular formula	Error (ppm)	MS/MS fragment ions	λ_{max} (nm)	Compound
7.14	**1**	449.1073	451.1230	451.1240	$C_{21}H_{23}O_{11}$	-2.2	231, 219, 195, 165	232, 288	(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside
8.42	**2**	449.1075	451.1238	451.1240	$C_{21}H_{23}O_{11}$	-0.4	231, 219, 195, 165	232, 287	(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside
9.52	**3**	449.1079	451.1239	451.1240	$C_{21}H_{23}O_{11}$	-0.2	231, 219, 195, 165	232, 286	(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside
10.10	**4**	449.1078	451.1226	451.1240	$C_{21}H_{23}O_{11}$	-3.2	231, 219, 195, 165	232, 286	(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside
10.67	**5**	325.0931	327.1071	327.1080	$C_{15}H_{19}O_8$	-2.9	119, 91	234, 282	*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)
12.52	**6**	447.0927	449.1079	449.1084	$C_{21}H_{21}O_{11}$	-1.1	329, 299	230, 269, 349	Iso-orientin
12.93	**7**	447.0922	449.1079	449.1084	$C_{21}H_{21}O_{11}$	-1.1	329, 299	230, 267, 347	Orientin
14.38	**8**	451.1235	453.1393	453.1397	$C_{21}H_{25}O_{11}$	-0.9	333, 247, 235, 205, 193, 165, 151, 123	230, 288	Aspalathin
17.25	**9**	609.1473	611.1600	611.1612	$C_{27}H_{31}O_{16}$	-2.0	303	256, 350	Quercetin-3-*O*-robinobioside
17.96	**10**	431.0994	433.1135	433.1135	$C_{21}H_{21}O_{10}$	0.0	313, 283	238, 268, 338	Vitexin
18.74	**11**	463.0873	465.1037	465.1033	$C_{21}H_{21}O_{12}$	0.9	303	256, 355	Hyperoside
19.15	**12**	609.1448	611.1601	611.1612	$C_{27}H_{31}O_{16}$	-1.8	303	255, 351	Rutin
19.65	**13**	431.0978	433.1131	433.135	$C_{21}H_{21}O_{10}$	-0.9	313, 283	239, 270, 338	Isovitexin
20.39	**14**	463.0863	465.1039	465.1033	$C_{21}H_{21}O_{12}$	1.3	303	256, 355	Isoquercitrin
21.51	**15**	447.0927	449.1087	449.1084	$C_{21}H_{21}O_{11}$	0.7	287.	238, 280, 340	Luteolin-7-*O*-glucopyranoside
24.47	**16**	435.1298	437.1448	437.1448	$C_{21}H_{25}O_{10}$	0.0	247, 193, 107	237, 286	Nothofagin

3.2. Method Validation

The first step in method validation was to confirm the peak identities. This was achieved by hyphenation of the optimised method to ESI-Q-TOF detection. MS results confirmed that no co-elution occurred using the new method. The MS data (**Table 3.3**) compared favourably with those reported by Beelders *et al.*, (2012). A similar elution order was also observed for rooibos phenolics in the current study, confirming that the superficially porous phase displayed roughly equivalent selectivity to the fully porous C_{18} phase. Phenolic compounds can be tentatively identified with a relatively high degree of certainty based on their accurate mass information and MS fragmentation behaviour and extensive structural elucidation guidelines reported in literature (Abad-Garcia *et al.*, 2008; Abad-Garcia *et al.*, 2009). For compounds **5**-**16**, fragmentation data were compared to literature reports (Abad-Garcia *et al.*, 2008; Iswaldi *et al.*, 2011; Beelders *et al.*, 2012), while comparison of retention times and mass spectral behaviour with authentic standards further confirmed the identification of these compounds, except for compound **9** (for which no reference standard was available).

The base peak intensity (BPI) chromatogram of a rooibos water extract (**Figure 3.3**) shows partial co-elution of compounds **9** and **10**, as well as **13** and **14**. This is due to differences in the delay volumes between the HPLC and UPLC instruments, as well as unavoidable extra-column band broadening due to the connection tubing between the DAD and MS detectors. These differences were partially compensated for by increasing the temperature to 47 °C. Mass spectral data were sufficient to confirm peak purity and identity (**Table 3.3**).

The four tentatively identified flavanones (compounds **1**-**4**) displayed identical MS behaviour, as reported previously for four eriodictyol-*C*-β-D-glucopyranoside isomers (Kazuno *et al.*, 2005). Compounds **1** and **2** were identified as eriodictyol-6-*C*-β-D-glucopyranoside isomers based on the fact that both lose a water molecule more readily than compounds **3** and **4** in positive ionisation mode, leading to higher abundances of the $[M+H-H_2O]^+$ ions, in accordance with Abad-Garcia *et al.*, (2008). The $[M+H-150]^+$ ion was twice as abundant as the $[M+H-120]^+$ ion for compounds **1** and **2**, whereas a ratio of about 1:1 was observed for these two ions in the case of compounds **3** and **4** (Abad-Garcia *et al.*, 2008). Furthermore, the peak area ratios for compounds **1** and **2** relative to those of **3** and **4** were roughly 2:1, in accordance with the more favourable formation of the 6-*C*-eriodictyol-glucopyranosides over their -8-*C* counterparts (Krafczyk & Glomb, 2008; Beelders *et al.*, 2012). Based on these observations, and reported RP-HPLC elution orders of eriodictyol glucopyranoside isomers (Bramati *et al.*, 2002; Abad-Garcia *et al.*, 2008; Krafczyk & Glomb, 2008; Beelders *et al.*, 2012), compounds **1** and **2** were identified as (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside, respectively, and compounds

3 and **4** as (*S*)-eriodictyol-8-*C*-β-D-glucopyranoside and (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside, respectively.

The method was deemed specific for the sixteen peaks selected for quantification as discussed above. The linearity of the calibration curves (8 levels) was excellent, with correlation coefficients larger than 0.9996 (**ADDENDUM A**, **Table A.1**). The y-intercept values were also relatively low. The stability of the phenolic compounds in the standard calibration mixture and unfermented and fermented sample extracts was very good over a 24 h period (% RSD < 5%; **ADDENDUM A, Table A.2**). The repeatability of the method was excellent for most phenolic compounds (**ADDENDUM A**, **Table A.2**), with % RSDs between 0.1 and 5%. The exceptions were (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside and hyperoside in the fermented extract, which were characterised by % RSD values just above 5%. These were deemed acceptable, given the complexity of the sample matrix.

3.3. Optimisation of Rooibos Plant Material Extraction

In order to determine the best solvent for the extraction of phenolics from rooibos plant material, green, semi-fermented and fermented rooibos samples were extracted to accommodate changes in the matrix with fermentation. Water, acetonitrile, ethanol and acetonitrile-water and ethanol-water mixtures were employed. Compounds were quantified in the different extracts, and subjected to multivariate data analysis using PCA. Mean centering was performed prior to data analysis, as large differences in the quantitative phenolic composition between the three types of plant materials were evident. A PCA bi-plot (**Figure 3.4**) constructed by using the mean centered data shows that most of the variation in the data can be explained by the first factor (86.8%). The compounds were clustered to the right of the plot and associated with all three plant material types extracted using 40% acetonitrile, as well as fermented plant material extracted with 80% ethanol and semi-fermented plant material extracted with 60% acetonitrile. Based on these results, 40% acetonitrile was selected as the preferred extraction solvent for phenolic compounds from green, semi-fermented and fermented rooibos plant material.

ANOVA was also performed on the actual phenolic content values for the green, semi-fermented and fermented rooibos plant material separately (**ADDENDUM A**, **Table A.3-A.5**). Extraction using 40% acetonitrile and 80% ethanol gave similar results for most compounds in the green plant material. The exceptions were significantly ($p < 0.05$) lower values for (*R*)-eriodictyol-6-*C*-glucopyranoside, PPAG and orientin when extracting with 40% acetonitrile and significantly ($p < 0.05$) lower values for (*S*)-eriodictyol-6-*C*-glucopyranoside and (*R*)- and (*S*)-eriodictyol-6-*C*-glucopyranoside for 80% ethanol. In the case of the semi-fermented plant material, 60% acetonitrile provided the best extraction, while in many cases compound values were not significantly ($p \geq 0.05$) different compared to those obtained for

40% acetonitrile extraction. The highest values from fermented rooibos plant material were obtained with 80% ethanol, although in many cases values for 40% acetonitrile did not differ significantly ($p \geq 0.05$). These results confirm the choice of 40% acetonitrile as extraction solvent.

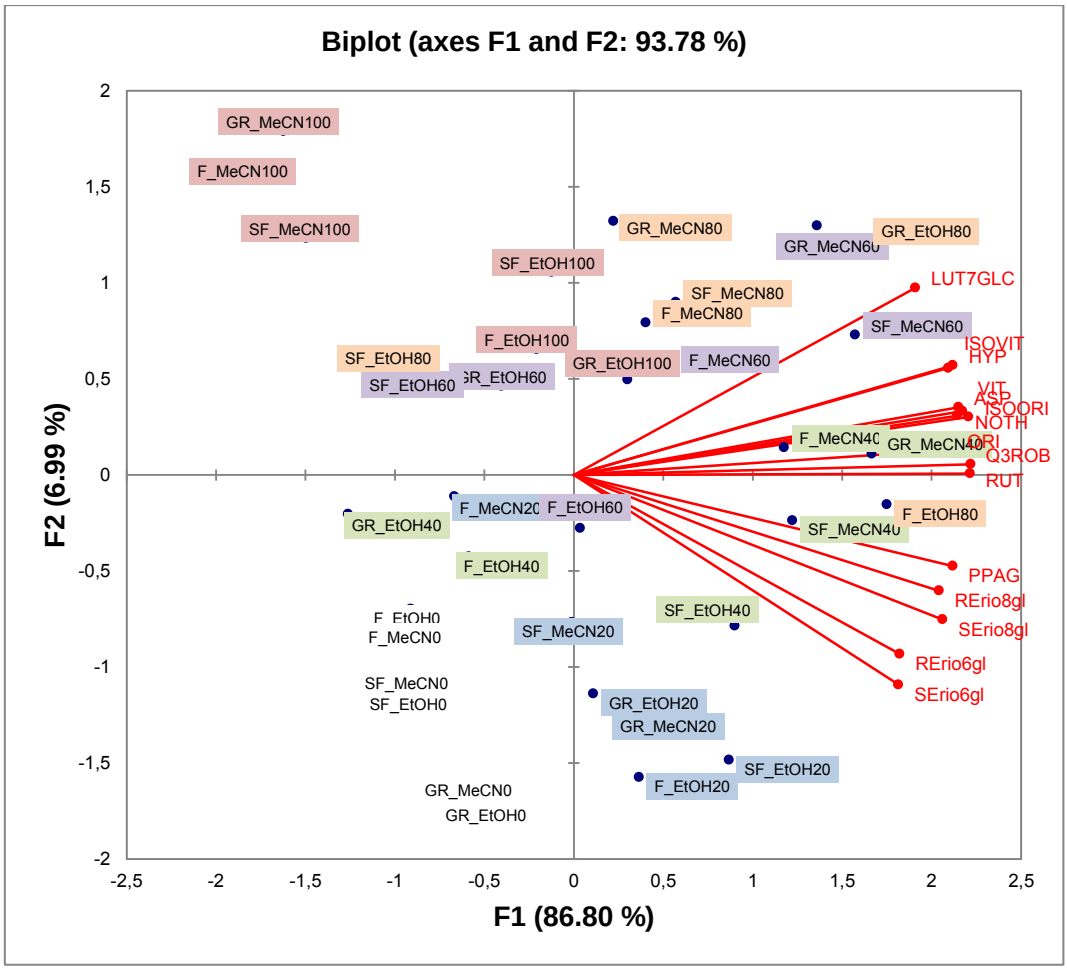


Figure 3.4 Principle component analysis of mean centred polyphenolic composition data obtained using water, 20%, 40%, 60%, 80% and 100% ethanol and acetonitrile independently as extraction solvents for rooibos plant material. Each score represents the average of three replicate extractions. *Abbreviations:* ASP, aspalathin; EtOH, ethanol; F, fermented; GR, green; HYP, hyperoside; ISOORI, iso-orientin; ISOVIT, isovitexin; LUT7GLC, luteolin-7-*O*-β-D-glucopyranoside; MeCN, acetonitrile; ORI, Orientin; PPAG, *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid; Q3ROB, quercetin-3-*O*-robinobioside; RErio6gl, (R)-eriodictyol-6-C-β-D-glucopyranoside; RErio8gl, (R)-eriodictyol-8-C-β-D-glucopyranoside; RUT, Rutin; SErio6gl, (S)-eriodictyol-6-C-β-D-glucopyranoside; SErio6gl, (S)-eriodictyol-8-C-β-D-glucopyranoside; SF, semi-fermented; NOTH, nothofagin; VIT, vitexin.

3.4. Effect of Fermentation on Rooibos Phenolic Composition

Ten green, semi-fermented and fermented paired-processed rooibos samples were analysed to determine the effect of fermentation on the quantitative phenolic composition of rooibos. A hot water extract of each sample was analysed (**Table 3.4**) as it represents the most common food ingredient extract (values expressed as g.100 g^{-1} extract) produced from rooibos tea and should provide information similar to that of a "cup of tea". In order to compare the plant material, extracts prepared using the optimal extraction solvent (40% aqueous acetonitrile) were analysed, as this extraction is less selective than a hot water extraction and therefore would represent the phenolic composition of the plant material (values expressed as g.100 g^{-1} plant material) (**Table 3.5**). This study is the first where the effect of fermentation was assessed using samples originating from the same bush, thereby minimising the contribution of factors other than the fermentation process such as the inherent variation in composition. The quantitative HPLC-DAD method was used to quantify 16 phenolic compounds present in the rooibos samples.

Table 3.4 Phenolic composition (g.100 g^{-1} extract; mean ± standard deviation) of green, semi-fermented and fermented rooibos hot water extracts

Compounds	Green (n = 10)	Semi-Fermented (n = 10)	Fermented (n = 10)
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]	nq[b]	$0.152 \pm 0.015_{B}$	$0.400 \pm 0.041_{A}$
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]	nq[b]	$0.148 \pm 0.014_{B}$	$0.398 \pm 0.043_{A}$
(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]	nq[b]	$0.092 \pm 0.017_{B}$	$0.139 \pm 0.024_{A}$
(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]	nq[b]	$0.080 \pm 0.006_{B}$	$0.131 \pm 0.018_{A}$
PPAG	$0.835 \pm 0.622_{A}$	$0.834 \pm 0.644_{A}$	$0.927 \pm 0.724_{A}$
Aspalathin	$8.411 \pm 0.998_{A}$	$4.280 \pm 0.877_{B}$	$1.366 \pm 0.419_{C}$
Nothofagin	$0.572 \pm 0.257_{A}$	$0.260 \pm 0.117_{B}$	$0.105 \pm 0.054_{C}$
Iso-orientin	$0.890 \pm 0.210_{A}$	$0.691 \pm 0.141_{B}$	$0.698 \pm 0.131_{B}$
Orientin	$0.820 \pm 0.181_{A}$	$0.643 \pm 0.113_{B}$	$0.815 \pm 0.130_{A}$
Quercetin-3-*O*-robinobioside[c]	$0.674 \pm 0.287_{A}$	$0.563 \pm 0.242_{B}$	$0.562 \pm 0.274_{B}$
Vitexin	$0.130 \pm 0.039_{A}$	$0.105 \pm 0.026_{B}$	$0.121 \pm 0.024_{AB}$
Hyperoside	$0.128 \pm 0.062_{A}$	$0.095 \pm 0.048_{B}$	$0.078 \pm 0.051_{B}$
Rutin	$0.246 \pm 0.100_{A}$	$0.212 \pm 0.092_{B}$	$0.161 \pm 0.085_{C}$
Isovitexin	$0.157 \pm 0.047_{A}$	$0.111 \pm 0.032_{B}$	$0.119 \pm 0.030_{B}$
Isoquercitrin	$0.092 \pm 0.047_{A}$	$0.064 \pm 0.033_{B}$	$0.070 \pm 0.037_{B}$
Luteolin-7-*O*-glucopyranoside	$0.061 \pm 0.027_{B}$	$0.071 \pm 0.035_{A}$	$0.057 \pm 0.027_{B}$

Different subscript capital letters indicate significant ($p < 0.05$) differences between treatments.

[a] Quantified as eriodictyol-7-*O*-glucopyranoside equivalents.

[b] Compounds not quantified due to low signal-to-noise ratios.

[c] Quantified as rutin equivalents.

Table 3.5 Phenolic composition (g.100 g^{-1} plant material; mean ± standard deviation)[a] of green, semi-fermented and fermented rooibos plant material extracted in 40% acetonitrile

Compounds	Green (n = 10)	Semi-Fermented (n = 10)	Fermented (n = 10)
(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]	nq[b]	$0.023 \pm 0.004_{B}$	$0.047 \pm 0.006_{A}$
(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]	nq[b]	$0.021 \pm 0.003_{B}$	$0.049 \pm 0.008_{A}$
(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]	nq[b]	$0.006 \pm 0.001_{B}$	$0.014 \pm 0.002_{A}$
(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]	nq[b]	$0.007 \pm 0.001_{B}$	$0.014 \pm 0.003_{A}$
PPAG	$0.100 \pm 0.083_{A}$	$0.081 \pm 0.064_{B}$	$0.083 \pm 0.060_{AB}$
Aspalathin	$1.201 \pm 0.034_{A}$	$0.610 \pm 0.139_{B}$	$0.170 \pm 0.077_{C}$
Nothofagin	$0.098 \pm 0.049_{A}$	$0.043 \pm 0.020_{B}$	$0.015 \pm 0.008_{C}$
Iso-orientin	$0.157 \pm 0.037_{A}$	$0.124 \pm 0.032_{B}$	$0.124 \pm 0.020_{B}$
Orientin	$0.149 \pm 0.034_{A}$	$0.120 \pm 0.028_{B}$	$0.118 \pm 0.018_{B}$
Quercetin-3-*O*-robinobioside[c]	$0.084 \pm 0.038_{A}$	$0.067 \pm 0.030_{B}$	$0.063 \pm 0.028_{B}$
Vitexin	$0.021 \pm 0.006_{A}$	$0.015 \pm 0.005_{B}$	$0.018 \pm 0.004_{B}$
Hyperoside	$0.016 \pm 0.009_{A}$	$0.011 \pm 0.007_{B}$	$0.012 \pm 0.009_{B}$
Rutin	$0.032 \pm 0.014_{A}$	$0.024 \pm 0.011_{B}$	$0.018 \pm 0.009_{B}$
Isovitexin	$0.034 \pm 0.010_{A}$	$0.025 \pm 0.008_{B}$	$0.023 \pm 0.006_{B}$
Isoquercitrin	$0.014 \pm 0.007_{A}$	$0.009 \pm 0.005_{B}$	$0.011 \pm 0.005_{B}$
Luteolin-7-*O*-glucopyranoside	$0.007 \pm 0.003_{B}$	$0.008 \pm 0.004_{B}$	$0.011 \pm 0.006_{A}$

Different subscript capital letters indicate significant (P<0.05) differences between treatments.

[a]Quantified as eriodictyol-7-*O*-glucopyranoside equivalents.

[b]Compounds not quantified due to low signal-to-noise ratios.

[c]Quantified as rutin equivalents.

The water extracts and plant material phenolic composition followed similar trends for the majority of the phenolic compounds (**Table 3.4** and **3.5**). Aspalathin and nothofagin, both *C*-glycosyl dihydrochalcones and major phenolic constituents of rooibos, were present at the highest concentrations in the green rooibos extracts and plant material. These compounds are known to undergo rapid degradation during fermentation (Joubert, 1996). The present data confirmed this, as evidenced by the substantial and significant ($p < 0.05$) decrease in their contents between green and semi-fermented (ca. 50%) and semi-fermented to fermented samples, with only 14-18% remaining in the fermented extract and plant material. Large variation observed between the different bushes is due to genetic variation, as rooibos is propagated from seeds. Previous studies have also demonstrated the large variation in phenolic content between rooibos bushes (De Beer *et al.*, 2016).

The eriodictyol-glucopyranosides, although detected, could not be quantified in the green rooibos water extracts and plant material due to the low amounts present. Only the sample used for method validation contained sufficient eriodictyol-glucopyranoside isomers for quantification. Their levels increased significantly ($p < 0.05$) in the semi-fermented and fermented samples. (*S*)- and (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside are formed via

oxidative cyclisation of aspalathin during fermentation (Marais *et al.*, 2000), while a subsequent Wessely-Moser rearrangement will result in (*S*)- and (*R*)-eriodictyol-8-*C*-β-D-glucopyranoside, respectively. The lower content of the 8-*C*-β-D-glucopyranoside isomers compared to the 6-*C*-β-D-glucopyranosides is explained by a slower rate of formation for the former (Krafczyk & Glomb, 2008). The corresponding intermediate oxidation products of nothofagin were not detected in rooibos, presumably due to very low amounts and possible co-elution with other peaks.

The iso-orientin and orientin content of the plant material decreased significantly ($p < 0.05$) from green to fermented samples, with no significant difference ($p \geq 0.05$) between the semi-fermented and fermented samples. Similarly, the iso-orientin content of the hot water extract decreased significantly ($p < 0.05$) from green to fermented rooibos. However, the orientin content of the hot water extracts did not follow this trend: the content of this compound decreased significantly ($p < 0.05$) from green to semi-fermented, and increased significantly ($p < 0.05$) again to fermented samples. The reason for this is not evident, based on the expected behaviour of the compound and its content in the plant material. One possible reason for the differences observed for orientin in the hot water extracts may be related to changes in the plant matrix, which affect the extractability of the compounds after fermentation (cf. Section 3.3). Degradation of the extract sample due to poor storage conditions is not likely, as samples were stored sealed under cool, dark, dry conditions until analysis. Furthermore, iso-orientin followed the expected pattern in the hot water extracts. Nevertheless, since the net effect was a decrease in the content of these two compounds, it can be concluded that the degradation reactions involving both proceed faster than the formation reactions.

Vitexin and isovitexin are both oxidation products of nothofagin (Joubert, 1996). Therefore, the low amounts of vitexin and isovitexin present in the samples can be attributed to the low levels of nothofagin originally present in the green plant material. The small but significant ($p < 0.05$) decreases observed for these two compounds in the hot water extract and plant material after fermentation can be explained by lower rates of formation via oxidation of nothofagin than their oxidation rates.

The PPAG content showed no significant differences ($p \geq 0.05$) between green and fermented treatments for both the hot water extract and plant material, indicating that this compound is not degraded under the conditions present during fermentation (exposure to moisture and air at 38 °C for 12 h).

The levels of the flavonols, namely quercetin-3-*O*-robinobioside, rutin, hyperoside and isoquercitrin, in both the hot water extracts and plant material, decreased as a result of fermentation. Some compounds were not yet significantly ($p < 0.05$) reduced in the semi-fermented samples. However, degradation of the flavonols (< 50%) from green to fermented

plant material was much less pronounced than for the dihydrochalcones. The content of the flavone luteolin-7-*O*-glucopyranoside significantly ($p < 0.05$) increased from green to semi-fermented, and decreased from semi-fermented to fermented hot water extracts. For the plant material the levels of this compound only decreased following fermentation. As the compound was present in very low quantities, the contradictory trends could be the result of difficulty in accurate peak integration.

From the above discussion, it is clear that flavanones, dihydrochalcones and flavonols follow distinct trends during the fermentation process. The flavones, however, displayed slightly different, less predictable trends in their quantitative data when compared to other rooibos flavonoids. This might be as a result of the interplay between degradation reactions involving these compounds during fermentation on the one hand, and their formation following oxidative degradation of other flavonoids on the other. It is important to note that as each set of green, semi-fermented and fermented samples originated from a single bush, it means that the effects observed can be directly related to the fermentation treatment, and not genetic variation between bushes.

Semi-fermented rooibos is not produced commercially. However, poor processing and storage conditions of green rooibos can lead to partial fermentation and thus deterioration of quality, observed as browning (Joubert & De Beer, 2011). The present study showed that partial fermentation will also result in substantial losses of the dihydrochalcones.

4. Conclusions

A new HPLC method utilising a 2.7 µm superficially porous stationary phase was developed and validated for the quantitative analysis of 16 rooibos phenolic compounds, including four eriodictyol-glucopyranoside isomers, which are oxidation products of aspalathin. This method therefore enables further study of the oxidation of aspalathin. The optimal solvent for the extraction of phenolic compounds from rooibos plant material was determined to be 40% aqueous acetonitrile, as this solvent provides the best overall extraction from green, semi-fermented and fermented samples. The levels of the majority of the extracted rooibos phenolic compounds decreased during the fermentation process, although concentrations of compounds such as the eriodictyol-glucopyranoside isomers and orientin increased. This can be explained by the fact that these compounds are formed as a consequence of the oxidation of aspalathin during fermentation.

References

Abad-Garcia, B., Berrueta, L. A., Garmon-Lobato, S., Gallo, B. & Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *Journal of Chromatography A,* **1216**, 5398-5415.

Abad-Garcia, B., Garmon-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2008). New features on the fragmentation and differentiation of *C*-glycosidic flavone isomers by positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry,* **22**, 1834-1842.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012). Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation. *Journal of Chromatography A,* **1219**, 128-139.

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P. & Pietta, P. (2002). Quantitative characterization of flavonoid compounds in rooibos tea (*Aspalathus linearis*) by LC-UV/DAD. *Journal of Agricultural and Food Chemistry,* **50**, 5513-5519.

De Beer, D., Miller, N. & Joubert, E. (2016). Production of dihydrochalcone-rich green rooibos (*Aspalathus linearis*) extract taking into account seasonal and batch-to-batch variation in phenolic composition of plant material. *South African Journal of Botany,* 10.1016/j.sajb.2016.02.198.

González-Ruiz, V., Olives, A. I. & Martín, M. A. (2015). Core-shell particles lead the way to renewing high-performance liquid chromatography. *Trends in Analytical Chemistry,* **64**, 17-28.

Hayes, R., Ahmed, A., Edge, T. & Zhang, H. (2014). Core-shell particles: preparation, fundamentals and applications in high performance liquid chromatography. *Journal of Chromatography A,* **1357**, 36-52.

Heinrich, T., Willenberg, I. & Glomb, M. A. (2012). Chemistry of color formation during rooibos fermentation. *Journal of Agricultural and Food Chemistry,* **60**, 5221-5228.

Iswaldi, I., Arraez-Roman, D., Rodriguez-Medina, I., Beltran-Debon, R., Joven, J., Segura-Carretero, A. & Fernandez-Gutierrez, A. (2011). Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Analytical and Bioanalytical Chemistry,* **400**, 3643-3654.

Joubert, E. (1996). HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chemistry,* **55**, 403-411.

Joubert, E. & De Beer, D. (2011). Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany,* **77**, 869-886.

Joubert, E. & De Beer, D. (2012). Phenolic content and antioxidant activity of rooibos food ingredient extracts. *Journal of Food Composition and Analysis,* **27**, 45-51.

Joubert, E., De Beer, D., Hernández, I. & Munné-Bosch, S. (2014). Accummulation of mangiferin, isomangiferin, iriflophenone-3-C-β-glucoside and hesperidin in honeybush leaves (Cyclopia genistoides Vent.) in response to harvest time, harvest interval and seed source. *Industrial Crops and Products,* **56**, 74-82.

Joubert, E., Gelderblom, W. C. A., Louw, A. & De Beer, D. (2008). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Journal of Ethnopharmacology,* **119**, 376-412.

Kazuno, S., Yanagida, M., Shindo, N. & Murayama, K. (2005). Mass spectrometric identification and quantification of glycosyl flavonoids, including dihydrochalcones with neutral loss scan mode. *Analytical Biochemistry,* **347**, 182-192.

Krafczyk, N. & Glomb, M. A. (2008). Characterization of phenolic compounds in rooibos tea. *Journal of Agricultural and Food Chemistry,* **56**, 3368-3379.

Malgas, R. R., Potts, A. J., Oettlé, N. M., Koelle, B., Todd, S. W., Verboom, G. A. & Hoffman, M. T. (2010). Distribution, quantitative morphological variation and preliminary molecular analysis of different growth forms of wild rooibos (*Aspalathus linearis*) in the northern Cederberg and on the Bokkeveld Plateau. *South African Journal of Botany,* **76**, 72-81.

Marais, C., Janse van Rensburg, W., Ferreira, D. & Steenkamp, J. A. (2000). (*S*)- and (*R*)-Eriodictyol-6-*C*-β-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*). *Phytochemistry,* **55**, 43-49.

Muller, C. J., Malherbe, C. J., Chellan, N., Yagasaki, K., Miura, Y. & Joubert, E. (2016). Potential of Rooibos, its Major *C*-Glucosyl Flavonoids and *Z*-2-(beta-D-Glucopyranoloxy)-3-phenylpropenoic acid in Prevention of Metabolic Syndrome. *Critical Reviews in Food Science and Nutrition,* 10.1080/10408398.2016.1157568.

Pasrija, D. & Anandharamakrishnan, C. (2015). Techniques for extraction of green tea polyphenols: A review. *Food and Bioprocess Technology,* **8**, 935-950.

Shabir, G. A., John Lough, W., Arain, S. A. & Bradshaw, T. K. (2007). Evaluation and application of best practice in analytical method validation. *Journal of Liquid Chromatography & Related Technologies,* **30**, 311-333.

Snyder, L. R., Kirkland, J. J. & Glajch, J. L. (1997). The Column. In: Practical HPLC Method Development. Pp. 174-232. John Wiley & Sons, Inc.

Chapter 4

Phenolic profiling of rooibos using off-line comprehensive two-dimensional normal phase countercurrent chromatography × reversed phase liquid chromatography with UV and mass spectrometric detection

Abstract

Limited performance of one dimensional (1D) chromatography techniques with regards to complex samples creates the incentive for the development of multidimensional chromatography techniques. Rooibos (*Aspalathus linearis*) represents such a complex sample, which contains a range of phenolic compounds that cannot be separated and identified with a single chromatographic technique within a reasonable time. Implementing normal phase (NP) high performance countercurrent chromatography (HPCCC) as the first dimension (^{1}D) and reversed phase (RP) ultra high pressure liquid chromatography (UHPLC) as the second dimension (^{2}D), a highly orthogonal (~80%) separation was achieved of the rooibos phenolic compounds in a total analysis time of 17 h. A high degree of orthogonality was achieved due to the differences in separation mechanisms, namely partitioning between two immiscible liquids for HPCCC and interaction with the solid stationary phase of functional groups associated with the solid stationary phase for UHPLC. Implementation of a gradient for the ^{1}D HPCCC separation ensured a good spread of relatively polar flavonoid di-*C*-glycosides and less polar mono- and di-*O*-glycosides, while the highly efficient UHPLC method was able to separate compounds eluting in the same ^{1}D fraction. Analysis of green and fermented rooibos samples enabled tentative identification of 39 phenolic compounds based on UV-Vis and mass spectrometric (MS) characteristics, 18 of which have not previously been reported in rooibos. Scolymoside (a flavone), hesperidin (a flavanone) and phloretin-3',5'-di-*C*-glucoside (a dihydrochalcone) were identified for the first time in rooibos by comparison with authentic reference standards. These compounds are also present in honeybush (*Cyclopia* spp.), which indicates the close relationship between these plants.

1. Introduction

The modern lifestyle can lead to lifestyle illnesses such as diabetes, chronic stress, obesity, weakening of the immune system and cardio-vascular disease (Van der Merwe *et al.*, 2006; Muller *et al.*, 2012; Oh *et al.*, 2013). In recent years, a large body of evidence has been gathered on the health-promoting effects of herbal teas (as reviewed by Da-Costa-Rocha *et al.*, 2014; Cardozo Junior & Morand, 2016; Lerotholi *et al.*, 2016). From the multitude of herbal teas available worldwide, rooibos (*Aspalathus linearis*) has received significant interest due to its associated health benefits (Sissing *et al.*, 2011; Kamakura *et al.*, 2015; Ku *et al.*, 2015a; Ku *et al.*, 2015b; Kwak *et al.*, 2015; Muller *et al.*, 2016).

Rooibos contains unique phenolic compounds that have been linked to the health-promoting benefits of the product (Muller *et al.*, 2016). Quantification and identification of the phenolic compounds in rooibos underpins research on the bioactivity of rooibos products. To date the most commonly used method to analyse rooibos polyphenols is RP-HPLC, often hyphenated with mass spectrometry (MS). In order to accurately quantify a compound using liquid chromatography with non-specific detection, it must be separated from other compounds present in the sample matrix. This can be difficult as rooibos samples, like most natural products, contain a multitude of polyphenols. A large number of isomers, such as regioisomers and stereoisomers, contribute to the difficulty of separation using reversed phase liquid chromatography (RP-LC). High resolution MS and/or high energy tandem mass spectrometry (MS/MS) fragmentation patterns and intensity data can be used in conjunction with UV-Vis data to tentatively identify isomers (Beelders *et al.*, 2012c; De Villiers *et al.*, 2016). However, the performance of one-dimensional (1D) chromatography is often not sufficient for highly complex samples (Kalili & De Villiers, 2013). One solution to this hurdle is to use multidimensional chromatography for increased performance (Kalili & De Villiers, 2013).

Countercurrent chromatography (CCC) is a popular preparative separation technique mostly used for the isolation of natural product compounds. This technique uses liquid stationary and mobile phases, referred to as the solvent system (refer to chapter 2 for further details on CCC). Both LC and CCC are 1D separation techniques and have been combined in previous research for two dimensional (2D) separation of phenolics (Michel *et al.*, 2011; Chen *et al.*, 2015). In 2D chromatography, a second separation dimension (^{2}D) is used to further resolve compounds that have been initially separated in a ^{1}D. It is important that the two separations must be orthogonal to obtain maximum overall performance (Gilar *et al.*, 2005); from this perspective the combination of CCC and RP-LC is particularly promising.

The aim of this study was to develop and apply an analytical scale off-line comprehensive 2D separation method in order to gain detailed qualitative information on the

phenolic composition of green and fermented rooibos extracts. The ^{1}D was a NP high performance CCC (HPCCC) analysis followed by the ^{2}D RP-ultra-high performance LC (UHPLC) analysis. To confirm peak purity and tentatively assign peaks, authentic reference standards, UV-Vis and time-of-flight MS (TOFMS) characteristics were interpreted.

2. Materials and Methods

2.1 Chemicals

Deionised water, prepared using a Millipore Elix Advantage (Continental Water Systems Corporation, San Antonio, TX, USA) water purification system, was further purified to HPLC-grade using a Milli-Q academic (Millipore, Bedford, USA) water purification system. Ethyl acetate (EtOAc) was purchased from Merck (Darmstadt, Germany) and gradient grade *n*-butanol (*n*-BuOH) from Sigma-Aldrich (St Louis, MO, USA). HPLC gradient grade acetonitrile was purchased from Merck for UHPLC analysis, while super purity acetonitrile (far UV) solvent was purchased from Romil (Cambridge, United Kingdom) for LC-MS analyses. Glacial acetic acid was purchased from Sigma-Aldrich. Authentic reference standards (purity ≥ 95%) were purchased from Extrasynthese (Genay, France; iso-orientin, orientin, eriodictyol-7-*O*-glucopyranoside, iso-vitexin, hyperoside, diosmetin-7-*O*-rutinoside, hesperidin), Phytolab (Vestenbergsgreuth, Germany; vicenin-2), Karl Roth (Karlsruhe, Germany; vitexin, luteolin-7-*O*-glucopyranoside), Sigma-Aldrich (isoquercitrin), Transmit (Gießen, Germany; rutin) and the PROMEC unit of the Medical Research Council of South Africa (MRC, Cape Town, South Africa; aspalathin and nothofagin). Phloretin-3',5'-di-*C*-glucoside (94% purity by LC-MS) and scolymoside (98% purity by LC-MS), were isolated from *C. subternata* (Post-Harvest and Wine Technology Division of ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). The polyphenolic standard stock solutions were prepared in dimethyl sulfoxide (DMSO) at concentrations of approximately 1 mg.mL^{-1} and diluted with water as required. All the diluted standard mixtures contained ca. 1% ascorbic acid (Sigma-Aldrich) and were filtered through 0.22 µm hydrophilic PVDF filters (Millipore) before use.

2.2. Sample Preparation

Hot water extracts were prepared from one selected green and fermented rooibos sample originating from the same bush as described in Chapter 3. In order to remove the ethanol (EtOH) insoluble fraction, 1 g of the water extract was mixed with 1 L 99.9% ethanol, followed by sonication for 30 min. The mixture was filtered through a Whattman #4 filter paper to separate the ethanol insoluble matter. Water was added to the filtrate and the ethanol was evaporated using rotary evaporation under vacuum, after which the ethanol

extract was freeze dried using a VirTis Genesis freeze dryer (SP Scientific, Warminster, PA, USA).

2.3. Instrumentation

The ^{1}D HPCCC separation was performed using a multilayer coil J-type centrifuge HPCCC spectrum model (dynamic extractions, Slough, UK) with analytical coils connected in series (polytetrafluorethylene (PTFE) tubing 0.8 i.d. 20.5 mL volume). The Beta Ratio (β_r) values for the analytical coil were measured as 0.64 at the internal end of the coil and 0.81 at the external end of the coil (β_r = r/R, with r = spool radius measured at the nearest and farthest layer of the coil; and R = rotor radius). The coils were connected from peripheral to centre (normal phase) with regard to flow direction. A 2.5 mL analytical injection loop was used. The average volume of the flying leads (4.0 m, i.d. 0.8 mm) per coil is 2.2 mL, giving a total volume of 4.4 mL for both coils.

Ultra High pressure Liquid Chromatography (UHPLC)-DAD analyses were conducted on an Agilent UHPLC 1290 Infinity instrument with a maximum pressure limit of 1200 bar (Agilent Technologies, Santa Clara, CA, USA). The 1290 Infinity series is equipped with intelligent system emulation technology (ISET), which was used to simulate the conditions of the Waters UPLC used for MS analyses (see next paragraph). The instrument was equipped with an online degasser, binary pump, autosampler, column thermostat containing a 3 µL solvent preheater, inline column filter (0.22 µm) and a Diode Array Detector (DAD). The UHPLC was controlled by Chemstation software (Agilent Technologies). The separation was performed using an Agilent Poroshell 120 SB-C_{18} column (100 x 2.1 mm, i.d. 1.8 µm) (Agilent Technologies). A maximum pressure setting of 600 bar was used as this is the maximum pressure limit of the Poroshell column.

Liquid Chromatography (LC)-MS analyses were conducted on a UPLC system coupled to a Synapt G2 quadrupole-time-of-flight (Q-TOF) mass spectrometer (Waters, Milford, USA) using a maximum pressure setting of 600 bar. The UPLC instrument was equipped with a binary solvent manager, sample manager, column heating compartment, pre-column heater and a DAD. The detector was equipped with a 500 nL flow cell. The ion source was an electrospray ionisation (ESI) unit.

2.4. High Performance Countercurrent Chromatography (HPCCC)-PDA Experimental Conditions

Initial method development for the ^{1}D CCC separation involved the isocratic elution of the rooibos ethanol soluble fractions injected at 10 mg with EtOAc:water (1:1) as the selected solvent system at a flow rate of 1 mL.min^{-1}. The lower layer (LL, aqueous phase) was

selected as the stationary phase and the upper layer (UL, organic phase) as the mobile phase, i.e. CCC was performed in NP mode. The same experiment was repeated using *n*-BuOH:water (1:1) as the solvent system instead of EtOAc:water (1:1). Both solvents systems were prepared by mixing equal volumes of organic solvent and water in a separation funnel, followed by phase separation after equilibration. The separated phases were degassed by sonication for 10 s. Prior to analysis the coils were filled with the stationary phase before rotation was started at 1600 rpm and the system was equilibrated at a temperature of 30 °C. The solvents reservoirs were placed in a temperature controlled water bath to maintain a constant solvent temperature of 30 °C.

Following the isocratic elution experiments, gradient elution was investigated. The LL of EtOAc:water (1:1) (A) was used as stationary phase and the UL from the same solvent system was used for the initial mobile phase (B). The secondary mobile phase solvent was the UL of *n*-BuOH:water (1:1) (C). The UL and LL of the two solvent systems were prepared separately as needed according to compositions reported by Garrard *et al.*, (2007). The initial elution gradient performed at 1 mL.min^{-1} using solvents B and C was: 0-14 min, 100% B; 14-70 min, 100-0% B. The extrusion step (solvent A at 1.5 mL.min^{-1}) was from 70-90 min.

The final HPCCC method utilised the following elution gradient with solvents B and C at 1.5 mL.min^{-1}: 0-9.33 min, 100%B; 9.33-46.67 min, 100-50%B. After the elution gradient, the extrusion step (46.67-60 min) was performed using 100% A at 3 mL.min^{-1}. Final sample loading for the fermented and green samples were 7 and 5 mg, respectively, dissolved in 1 mL of B (initial mobile phase) and 1 mL of A (stationary phase). The samples were sonicated for 10 min to ensure complete dissolution. UV spectra were recorded between 210-400 nm and chromatograms were constructed by summing the signals from each recorded wavelength. Fractions of the ^{1}D effluent were collected with a Gilson FC 204 (Gilson, Inc., Middleton, WI, USA) fraction collector using a sampling time of 1.25 min.

Equations 1, 2 and **3** where used to determine the stationary phase retention (S_f) in HPCCC.

$$V_E = V_D + V_M \quad (1)$$

$$V_S = V_C - V_M \quad (2)$$

$$S_f = \frac{V_S}{V_C} \times 100 \quad (3)$$

Where:

V_E = volume of stationary phase eluted, which is calculated from the elution time of the solvent front

V_D = dead volume, i.e. volume of flying leads

V_M = mobile phase volume

V_S = stationary phase volume
V_C = column volume

2.5. Preparation of Collected Fractions for ^{2}D Analysis

The collected fractions were evaporated in a SpeedVac system (Thermo Scientific SPD 1010/SPD2010, Waltham, Massachusetts, USA) at 10 Torr and 45 °C. Continual heating for 6 h was used to evaporate all solvents.

The fractions were reconstituted with 20 µL DMSO, 10 µL 10% ascorbic acid and 600 µL distilled water and filtered through a 0.22 µm hydrophilic PVDF filters (Millipore) before use.

2.6. Ultra High Pressure Liquid Chromatography (UHPLC)-DAD Experimental Conditions

UHPLC-DAD analyses were performed using 2% aqueous acetic acid in water ($v.v^{-1}$) (Y) and acetonitrile (Z), both filtered through a 0.22 µm filter. The UHPLC flow rate was 0.8 $mL.min^{-1}$ and a multilinear gradient was used as follows: 10-14.8% Z (0-12.39 min), 14.8-19.2% Z (12.39-14.35 min), 19.2-100% Z (14.35-14.57min), 100% Z isocratic (14.57-16.52 min), 100-10% Z (16.52-17 min), 10% Z isocratic (17-20 min). UV spectra were recorded between 200-700 nm, with selected wavelength monitoring at 288 and 350 nm. The column temperature was set to 44.5 °C.

Intensity data between 250 and 400 nm were extracted and prepared using MATLAB R2015a (The MathWorks, Natick, USA). The maximum absorbance recorded between 250 and 400 nm was used to generate the contour plots in MATLAB.

2.7. Liquid Chromatography (LC)-MS Experimental Conditions

High Performance Countercurrent Chromatography (HPCCC) fractions were also analysed on a Waters UPLC-DAD-MS instrument using identical conditions as outlined above for UHPLC-DAD analyses. Similar separation was obtained compared to the UHPLC-DAD method, with only slight retention differences due to differences in instrument volumes.

The mass spectrometer was operated in positive ionisation mode. The eluent was split in a 1:1 ratio before entering the ionisation chamber. Mass calibration was performed using a sodium formate solution and leucine encephalin was used as the lockspray solution. A mass range of 150-1500 amu was scanned and MassLynx v.4.1 (Waters) software was used to process the data. The capillary voltage was 2.5 kV and the sampling cone voltage was set to 15 V. The source and desolvation temperatures were 120 °C and 275 °C, respectively. The desolvation and cone gas flows (N_2) were 650 and 50 $L.hr^{-1}$, respectively.

For both the MS^E and MS/MS experiments, the trap collision energy was ramped from 20.0 to 60.0 V.

2.8. Optimisation of CCC×LC Conditions

The practical peak capacity for the 2D separation was calculated using the equations described by Kalili & De Villiers, (2013). Different ^{1}D fraction collection times, ranging from 0.5 to 2 min, and ^{2}D analysis times, ranging from 5.0 to 30 min, were evaluated for method optimisation. The average peak width in the ^{1}D CCC separation was determined to be 2.3 min for the rooibos samples. Optimum ^{1}D sampling times and ^{2}D conditions were selected based on maximum practical peak capacity achievable as a function of total analysis time. **Equations 4-8** were used to determine the peak capacity for the ^{1}D, ^{2}D and the total 2D analysis, as well as the best ^{1}D sampling time and ^{2}D gradient time in order to gain the best possible peak capacity within reasonably total analysis time.

$$n = 1 + {}^{1}/_{\alpha} + \ln(V_n/V_1) \quad (4)$$

Where:

n = peak capacity

V_1 = minimum elution volume of the first peak

V_n = minimum elution volume of the nth peak

α = selectivity

$$n_{c,1D} = 1 + \frac{t_g}{\frac{1}{n}\sum_1^n w_b} \quad (5)$$

Where:

$n_{C,1D}$ = peak capacity of a comprehensive 2D separation

t_g = gradient time

ω_b = average peak width

The peak capacity of a comprehensive 2D separation can be determined according to the following equation:

$$n'_{c,2D} = \frac{{}^1n_c \times {}^2n_c}{\beta}. \quad (6)$$

Where:

$n'_{C,2D}$ = peak capacity of a comprehensive 2D separation

${}^{1}n_c$ = peak capacity of the ^{1}D separation

${}^{2}n_c$ = peak capacity of the ^{2}D separation

β = degree of under-sampling (Kalili & De Villiers, 2013)

$$\beta = \sqrt{1 + 3.35 \frac{{}^{1}t_s \times {}^{1}n_c}{{}^{1}t_g}} \quad (7)$$

Where:

${}^{1}t_s$ = ^{1}D sampling time

${}^{1}t_g$ = ^{1}D gradient time

$$t_{tot}, \text{off-line} = f \times {}^{2}t_c + f \times {}^{1}t_s \quad (8)$$

Where:

t_{tot}, off-line = total ^{2}D analysis time

f = total number of fractions sampled

${}^{2}t_c$ = ^{2}D cycle time

2.9. Determination of Orthogonality

Orthogonality was determined using conditional entropy (a discretised metric method) and convex hull (a non-discretised metric method) metrics. Conditional entropy was calculated using the procedure outlined by (Pourhaghighi *et al.*, 2011) (the MATLAB script for the calculation of conditional entropy script is presented in **ADDENDUM B, B.1**). Orthogonality according to the convex hull methods was determined according to (Semard *et al.*, 2010) (the MATLAB script is presented in **ADDENDUM B, B.2**). **Equation 9** was used to determine the total practical peak capacity by taking into account the effect of orthogonality.

$$n'_{c,2D} = \frac{{}^{1}n_c \times {}^{2}n_c \times f_c}{\beta} \quad (9)$$

Where:

$n'_{C,2D}$ = (practical) peak capacity of a comprehensive 2D separation

f_c = fractional surface coverage (= orthogonality%/100)

3. Results and Discussion

3.1. First Dimension CCC Method Development

Solvent system selection strategies for CCC have been extensively researched (Friesen & Pauli, 2005; Costa & Leitão, 2010; Costa & Leitao, 2011; Hopmann *et al.*, 2011; Liu *et al.*, 2015) as most CCC users experience difficulty when selecting a suitable solvent system. The solvent system family initially chosen for this study was based on previous reports on the separation of rooibos polyphenols using the EtOAc/*n*-BuOH/water (EBuWat) solvent system family (Krafczyk *et al.*, 2009; De Beer *et al.*, 2015). This solvent system family was therefore used as a starting point for the development of the ^{1}D separation method.

Unless stated otherwise, all HPCCC analyses were performed at a column temperature of 30 °C and the rotational speed of the HPCCC centrifugal unit was set to 1600 rpm. The instrument was filled with 100% of the selected stationary phase before the start of each analysis. The composition of the stationary phases used will be specified for each preliminary experiment in the following sections. The mobile phase and sample were introduced to the column simultaneously.

The first experiment was performed isocratically using a solvent system of 1:1 ethyl acetate and water. The LL was used as the stationary phase and the UL as the mobile phase, i.e. the NP mode was used. This resulted in the majority of compounds eluting in the coil fraction (**Figure 4.1**). The second experiment was carried out using a solvent system consisting of 1:1 *n*-BuOH and water, with the LL used as the stationary phase and the UL as the mobile phase. The captured fractions of both experiments were analysed by one dimensional HPLC-DAD using the method developed for quantitative rooibos analysis (chapter 3). It was observed that aspalathin, iso-orientin and orientin eluted earlier when using the *n*-BuOH:water system than the EtOAc:water system. Indeed, the majority of polyphenols (including those present at low quantities) had a shorter retention times when using the *n*-BuOH:water system (**Figure 4.2**). An extrusion step was added after 70 min for both isocratic systems, where 100% stationary phase (A) was pumped through the tubing, thereby pushing out the old stationary phase and phenolic compounds with a high affinity for A. The column was also brought to starting conditions upon completion of the extrusion step.

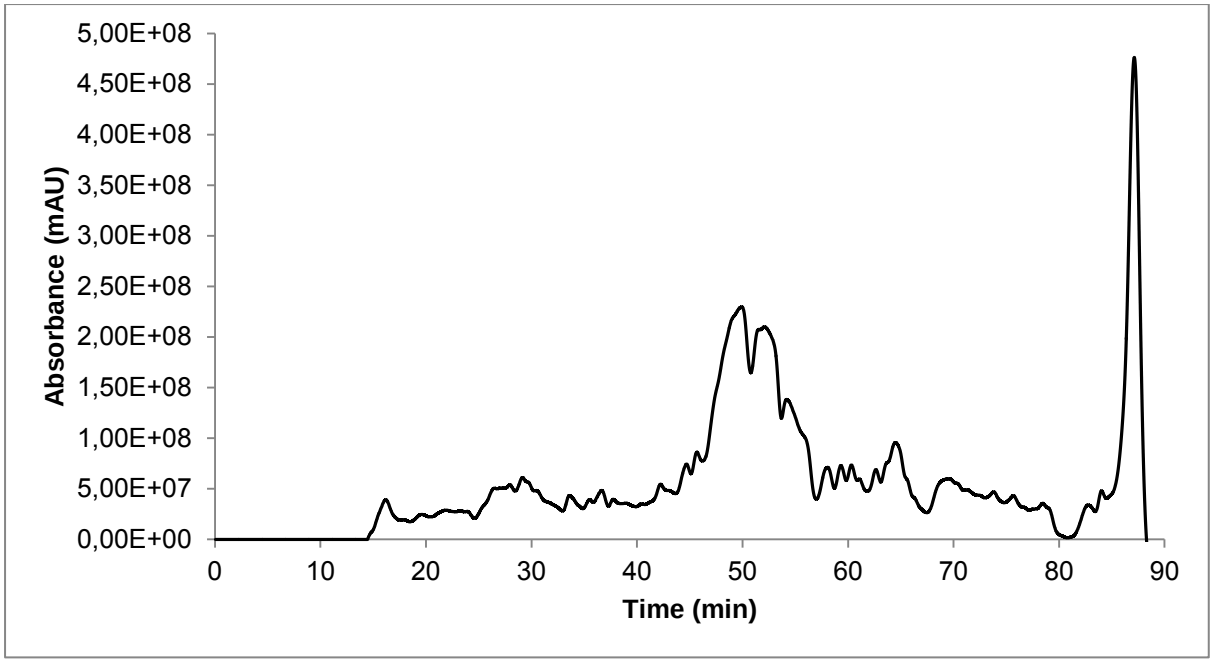


Figure 4.1 Isocratic normal-phase elution UV-Vis chromatogram (sum of signals between 210 and 400 nm) of the green rooibos extract (10 mg injected) using the ethyl acetate:water (1:1) solvent system (flow rate, 1 mL.min^{-1}; rotational speed, 1600 rpm; column temperature, 30 °C; extrusion step after 70 min at a flow rate of 1.5 mL.min^{-1}). The stationary phase retention was determined to be 63% using **equations 1-3**.

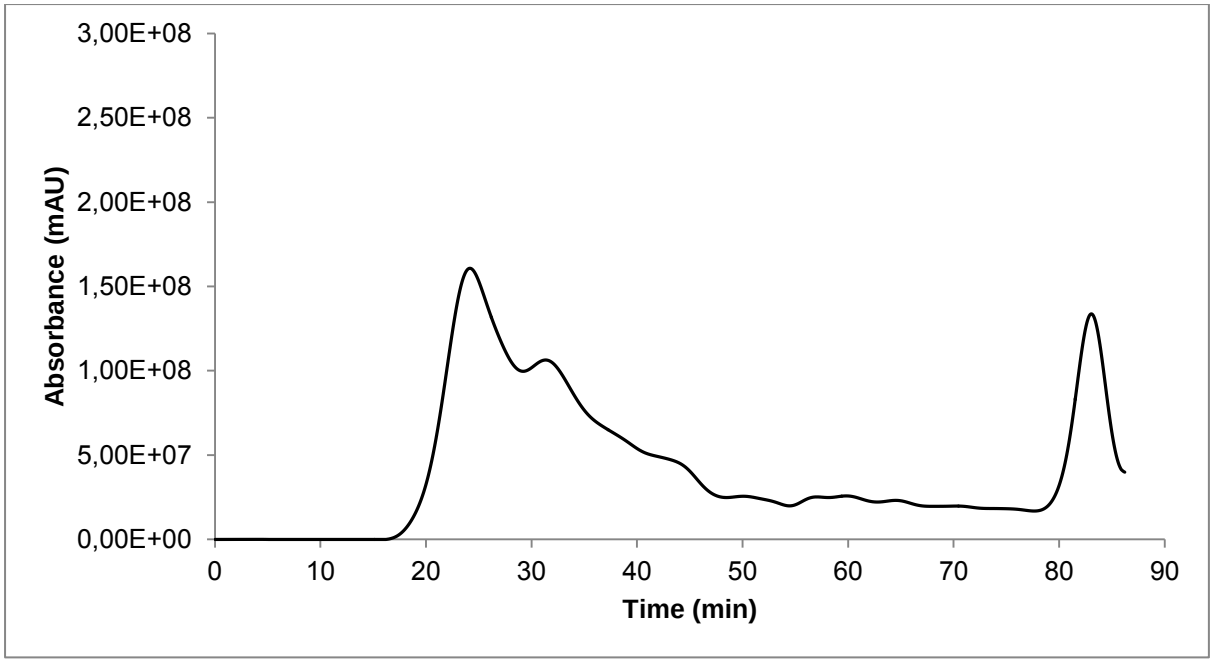


Figure 4.2 Isocratic NP elution UV-Vis chromatogram (sum of signals between 210 and 400 nm) of the green rooibos extract (10 mg injected) using the *n*-butanol:water (1:1) solvent system (flow rate, 1 mL.min^{-1}; rotational speed, 1600 rpm; column temperature, 30 °C; extrusion step, after 70 min with a flow rate of 1.5 mL.min^{-1}). The stationary phase retention was determined to be 58% using **equations 1-3**.

The previous analyses were performed using isocratic elution, meaning that the composition of the mobile and stationary phases did not change throughout the analysis. Adequate separation was not achieved under these conditions; therefore a gradient elution method using the EBuWat solvent system family was designed. Gradient elution in CCC is associated with inherent difficulties, as the composition of the stationary phase will undergo minor changes as the strength of the mobile phase increases during gradient elution (Leitao & Costa, 2015) (Refer to Chapter two for more information on gradient elution in CCC).

The stationary phase for the gradient elution analysis used the LL of EtOAc:water (1:1) (A) with the UL of the same solvent system (B) being the initial mobile phase. The elution started with an isocratic hold of 14 min at 100% B followed by a linear gradient elution from 100% B to 100% of the UL of *n*-BuOH:water (1:1) (C) over 70 min (see **Figure 4.3**). An extrusion step of 20 min was added after the gradient, where 100% of A was pumped through the coil. The gradient elution resulted in better spread of the compounds than isocratic elution, but the majority of the compounds eluted in the first half of the analysis. Therefore, the gradient was changed to start at 100% B and end at 50% C over 70 min, keeping the extrusion step the same as before. This resulted in better separation as the

compounds were more equally spread out over the chromatogram (data not shown). Finally, the total analysis time was reduced to 60 min, by increasing the mobile phase flow rate to 1.5 mL.min^{-1} for the elution step and 3 mL.min^{-1} for the extrusion step. These conditions resulted in the most acceptable separation within a reasonable time (see **Figure 4.4**).

Countercurrent chromatography is known for having higher sample loading capacity than conventional HPLC. This is due to a larger amount of stationary phase that is available to interact with sample compounds (Berthod *et al.*, 2009), although overloading can still occur. Sample overload can lead to loss of stationary phase, resulting in reduced resolution.

Total stationary phase loss was observed for the green and fermented rooibos extracts when injecting 10 mg into the column using the final ^{1}D conditions (data not shown). In order to determine whether stationary phase loss was due to emulsion formation, subsequent analyses were done at rotational speeds of 1200 and 1400 rpm. The change in rotational speed did not affect the loss of stationary phase. Since the problem seemed to stem from sample overloading, lower sample loadings were tested namely 7 mg of fermented rooibos extract and 5 mg of the green rooibos extract. Sample overloading was most probably caused by the high amounts of aspalathin present in the green rooibos extract (Joubert, 1996; Mekaoui *et al.*, 2012). Loss of stationary phase during the gradient elution in conjunction with increased mobile phase flow rate could also be a reason for the lower sample capacity compared to the initial isocratic analysis.

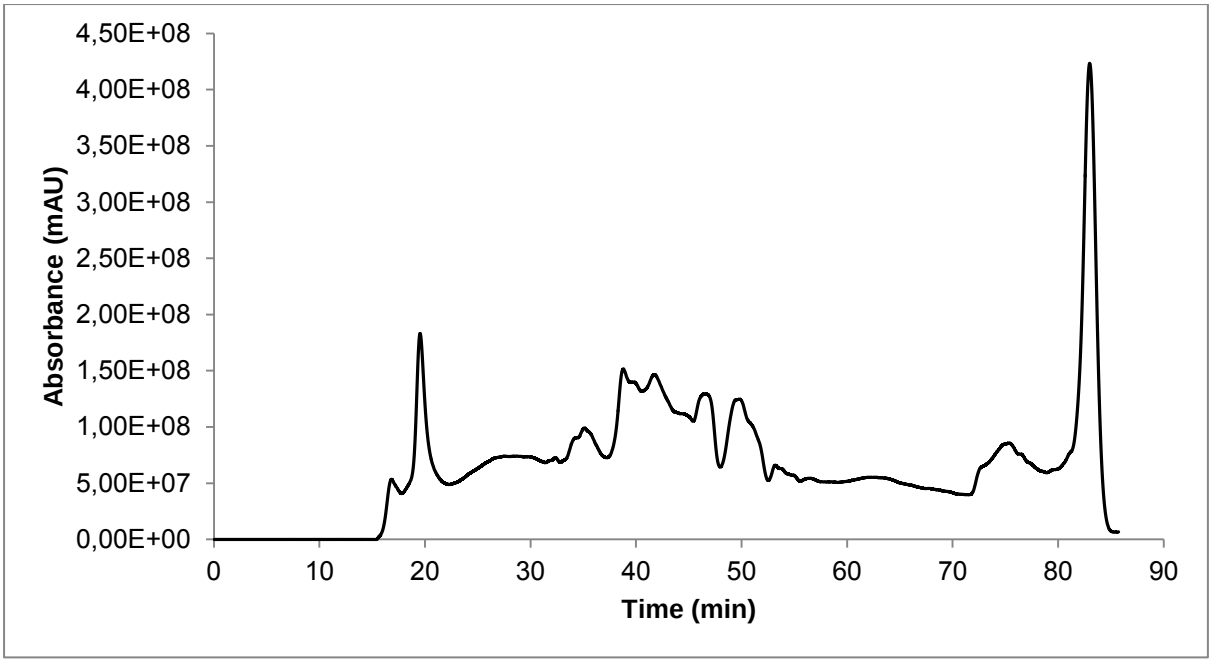


Figure 4.3 The UV-Vis chromatogram (sum of signals between 210 and 400 nm) for the green rooibos extract (10 mg injected), analysed using the initial NP gradient elution conditions (flow rate, 1 mL.min^{-1}; rotational speed, 1600 rpm; column temperature, 30 °C; extrusion step, after 70 min with a flow rate of 1.5 mL.min^{-1}; gradient using B (upper layer of 1:1 ethyl actetate:water) and C (upper layer of 1:1 *n*-butanol:water): 0-14 min, 100% B; 14-70 min, 100-0% B. The stationary phase retention was determined to be 60% using **equations 1-3**.

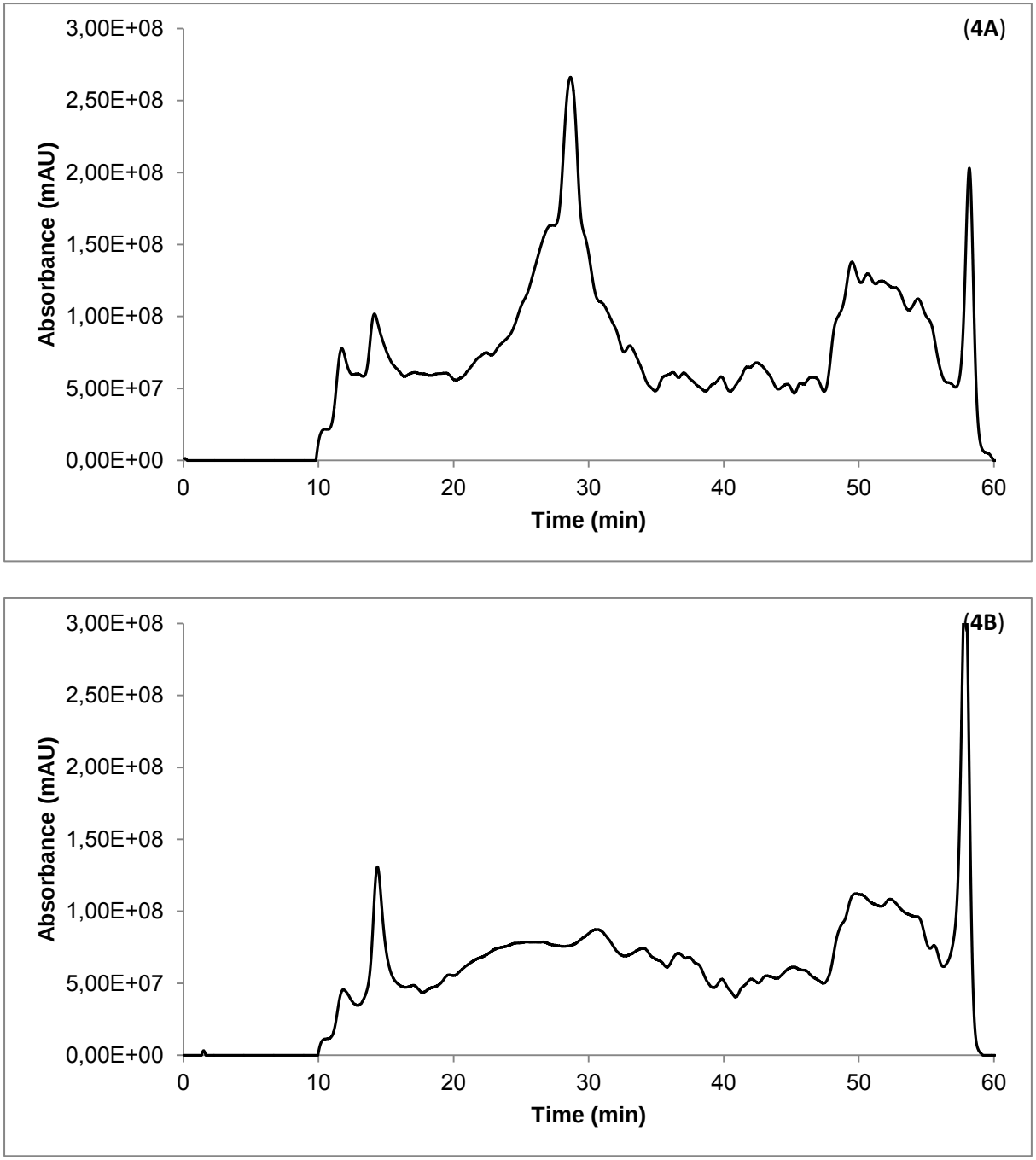


Figure 4.4 The UV-Vis chromatogram (sum of signals between 210 and 400 nm) (**4A**) for the green rooibos extract analysed using the final conditions developed for the ^{1}D (5 mg sample injected) and the (**4B**) fermented rooibos counterpart (7 mg sample injected) (flow rate, 1.5 mL.min^{-1}; rotational speed, 1600 rpm; column temperature, 30 °C; extrusion step, after 46.67 min with a flow rate of 3.0 mL.min^{-1}; gradient using B (upper layer of 1:1 ethyl actetate:water) and C (upper layer of 1:1 *n*-butanol:water): 0-9.33 min, 100% B; 9.33-46.67 min, 100-50% B). The stationary phase retention was determined to be 63% using **equations 1-3**.

3.2. Second Dimension UHPLC-DAD-MS Method Development

As multiple fractions were to be analysed in the ^{2}D, a rapid HPLC method was required. The performance of a superficially porous (Poroshell 120 SB-C_{18}, 100 x 2.1 mm i.d. 2.7 µm) and a fully porous sub-2 µm (ZORBAX SB-C_{18}, 100 x 2.1 mm i.d., 1.8 µm) column were compared for this purpose. The ZORBAX column has a maximum pressure of 1200 bar, while the Poroshell column can only support 600 bar. Each column was protected with a UHPLC guard column (ZORBAX SB-C_{18}, 5 x 2.1 mm i.d. 1.8 µm). The method reported in chapter 3 for quantitative analysis of rooibos phenolics was adjusted for the change in column dimensions and used as starting point for the ^{2}D method development.

In order to choose the most suitable stationary phase for a rapid method, the maximum flow rate that each column (with the guard column) could support was determined experimentally. The mobile phase composition at initial conditions for the gradient used here (10% B) had the highest viscosity, and therefore the max flow rate was determined under these conditions. The ZORBAX column could support a maximum flow of 0.7 mL.min^{-1} before the maximum pressure was reached, whereas the maximum flow rate for the Poroshell column was 0.8 mL.min^{-1}. This finding is in accordance with the larger particle size of the core-shell column (González-Ruiz *et al.*, 2015). The higher flow rate attainable on the Poroshell column is beneficial in the sense that shorter gradients can be used, and that less time is spent on column re-equilibration. Additionally, the Poroshell column provided higher resolution and peak capacity than the ZORBAX column under identical conditions. This observation is in line with theory, which predicts that core shell columns have similar performance to total porous column of smaller particle diameters. This is due to the narrow particle size distribution of core shell phases, possible roughness of the particles and the effect of this on packing efficiency, as well as a lower susceptibility to frictional heating. This column was therefore used for further experiments.

The performance of the Poroshell column was evaluated by measuring the peak capacity as a function of different gradient times (5, 7.5, 15, 20 and 30 min) and flow rates (0.45, 0.60, 0.80 mL.min^{-1}) (**Figure 4.5**). The peak capacity was determined according to **equation 4** for **5** different compounds (Giddings, 1967) at different gradient times and flow rates (**Figure 4.5**).

The highest peak capacity was achieved at a flow rate of 0.8 mL.min^{-1}, therefore further method development was performed at this flow rate. Different gradient times were tested (gradient time = 75% of cycle time). Bearing in mind the total analysis time of both dimensions, **equations 5** and **6** were used to determine the total peak capacity for the 2D separation. A cycle time of 20 min was chosen as the total peak capacity determined using **equation 6** was >3000 under these conditions (Neue, 2005; Beelders *et al.*, 2012a).

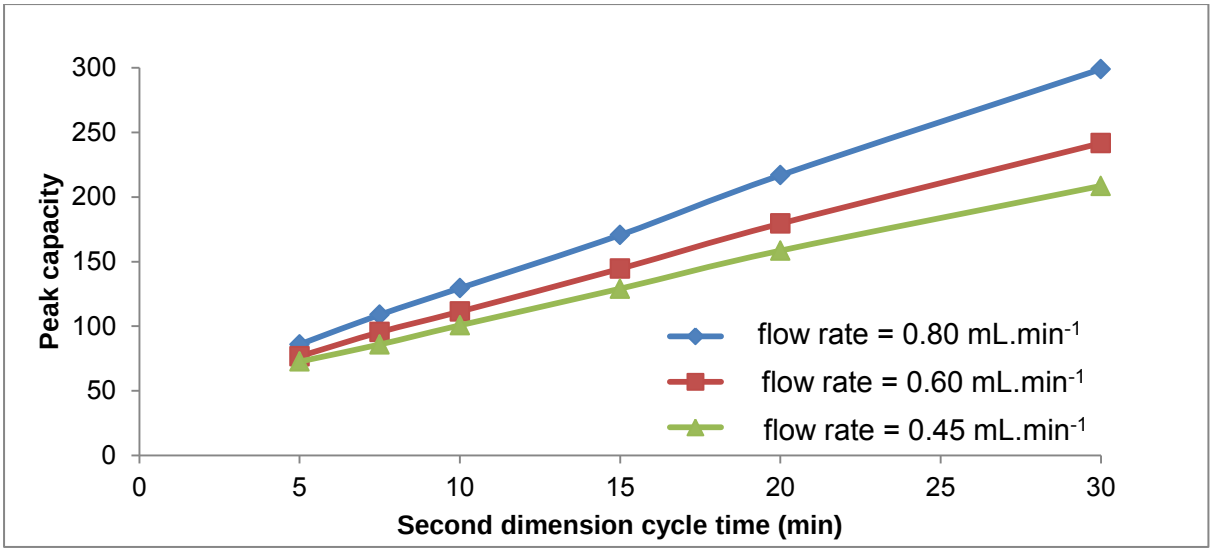


Figure 4.5 Peak capacity as a function of gradient time for the Poroshell 120 SB-C_{18} column at different flow rates.

The β of the ^{1}D peak is dependent on the $^{1}t_s$ and $^{1}t_g$ as seen in **equation 7**. As illustrated in **Figure 4.5** the total peak capacity is related to the ^{2}D gradient time. Finally, the total analysis time of an off-line comprehensive two-dimensional separation is determined according to **equation 8**.

Figure 4.6 illustrates the relationship between 2D peak capacity and total analysis time for three different sampling times and a fixed ^{2}D cycle time of 20 min, as determined using **equations 4-8**. Our goal was to obtain the high peak capacity (≈ 3000) for a reasonable total analysis time, considered to be ~1000 min. Based on these criteria, a ^{1}D sampling time of 1.25 min was selected, which provided a peak capacity value of 4085 for a total ^{2}D analysis time of 960 min (16 h) (**Figure 4.6**) and a total analysis time of 17 h.

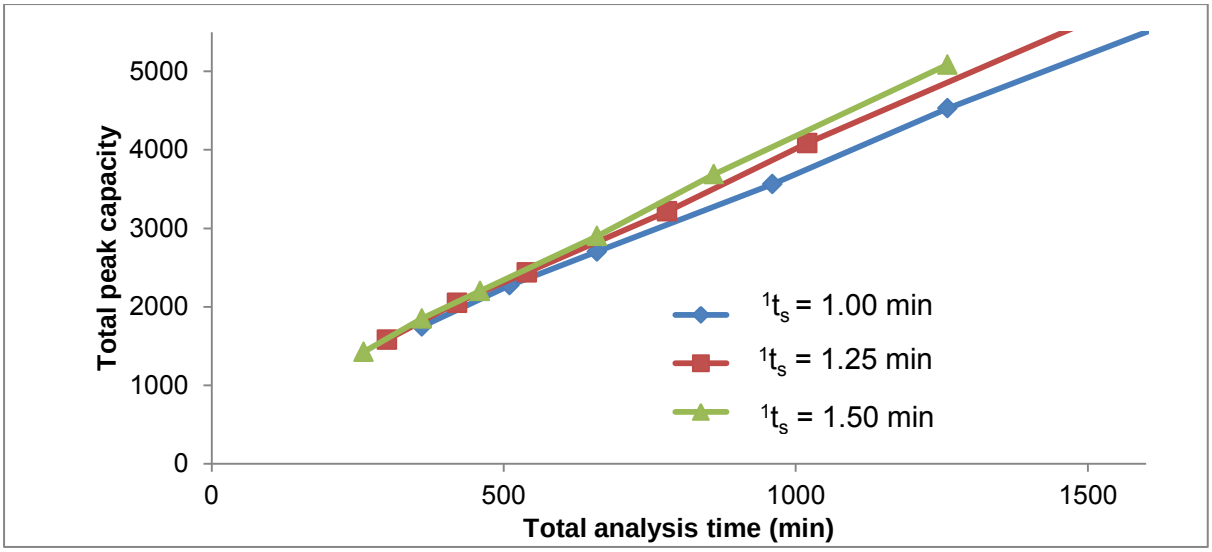


Figure 4.6 Relationship between the two-dimensional peak capacity and total analysis time for different ^{1}D sampling times using the following conditions: ^{1}D gradient time of 60 min with a elution flow rate of 1.5 mL.min^{-1} and an extrusion flow rate of 3.0 mL.min^{-1}.The (Poroshell 120 SB-C_{18}, 100 x 2.1 mm i.d. 2.7 µm) column was used for the ^{2}D with a flow rate of 0.8 mL.min^{-1} and a 20 min cycle time.

3.3. High Performance Countercurrent Chromatography (HPCCC)×UHPLC-DAD Analysis of Green and Fermented Rooibos and Evaluation of Method Performance

Green (n=1) and fermented (n=1) rooibos extracts were analysed by off-line HPCCC×UHPLC-DAD using the optimised experimental conditions as derived above. The corresponding DAD contour plots are presented **Figures 4.7** and **4.8**, respectively.

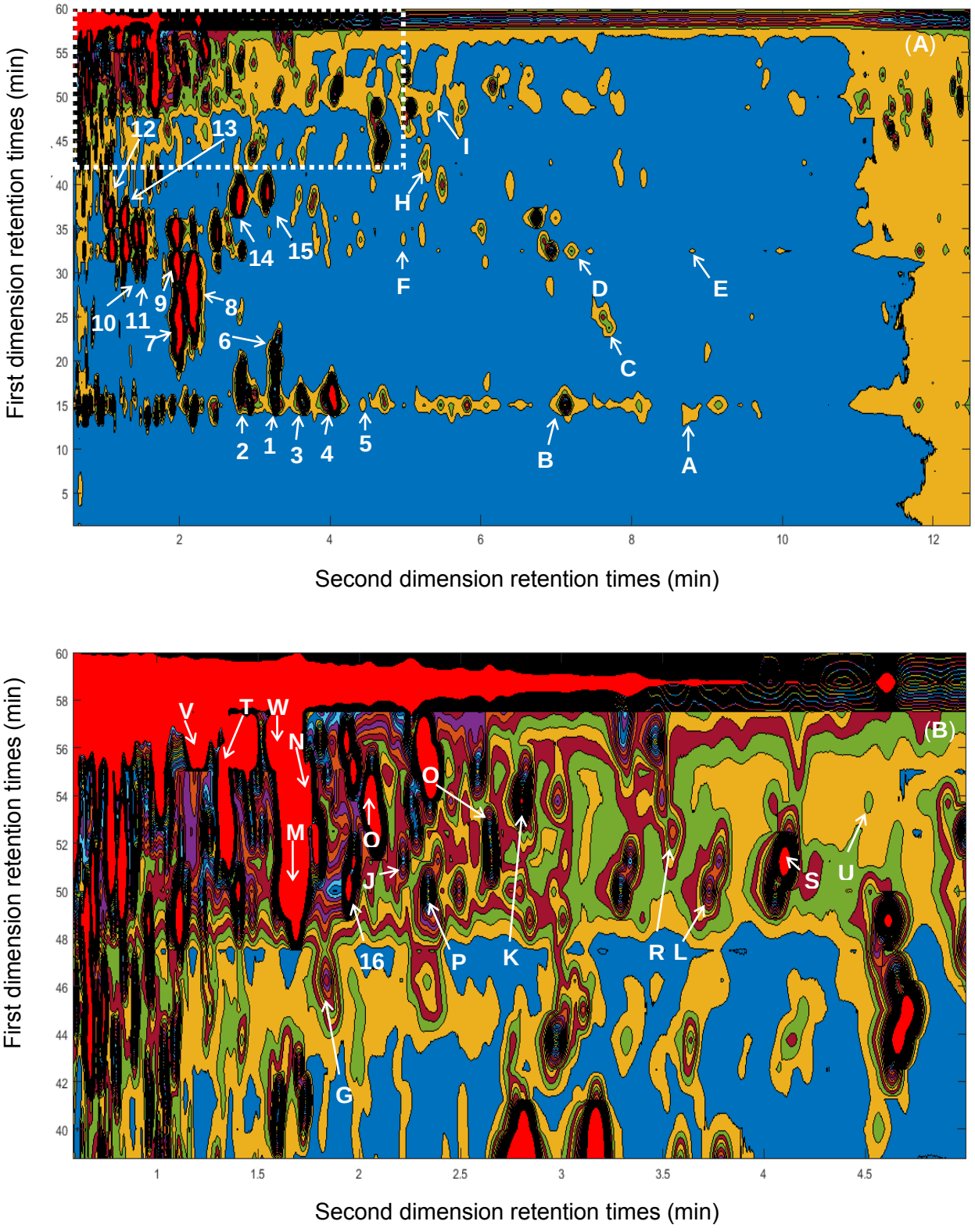


Figure 4.7 DAD contour plot representing the off-line HPCCC×RP-LC separation of a fermented rooibos extract (A), and zoomed view (corresponding to the dashed rectangular area in (A)) of the most polar compounds (B). Peak labels correspond to **Table 4.1-4.4**.

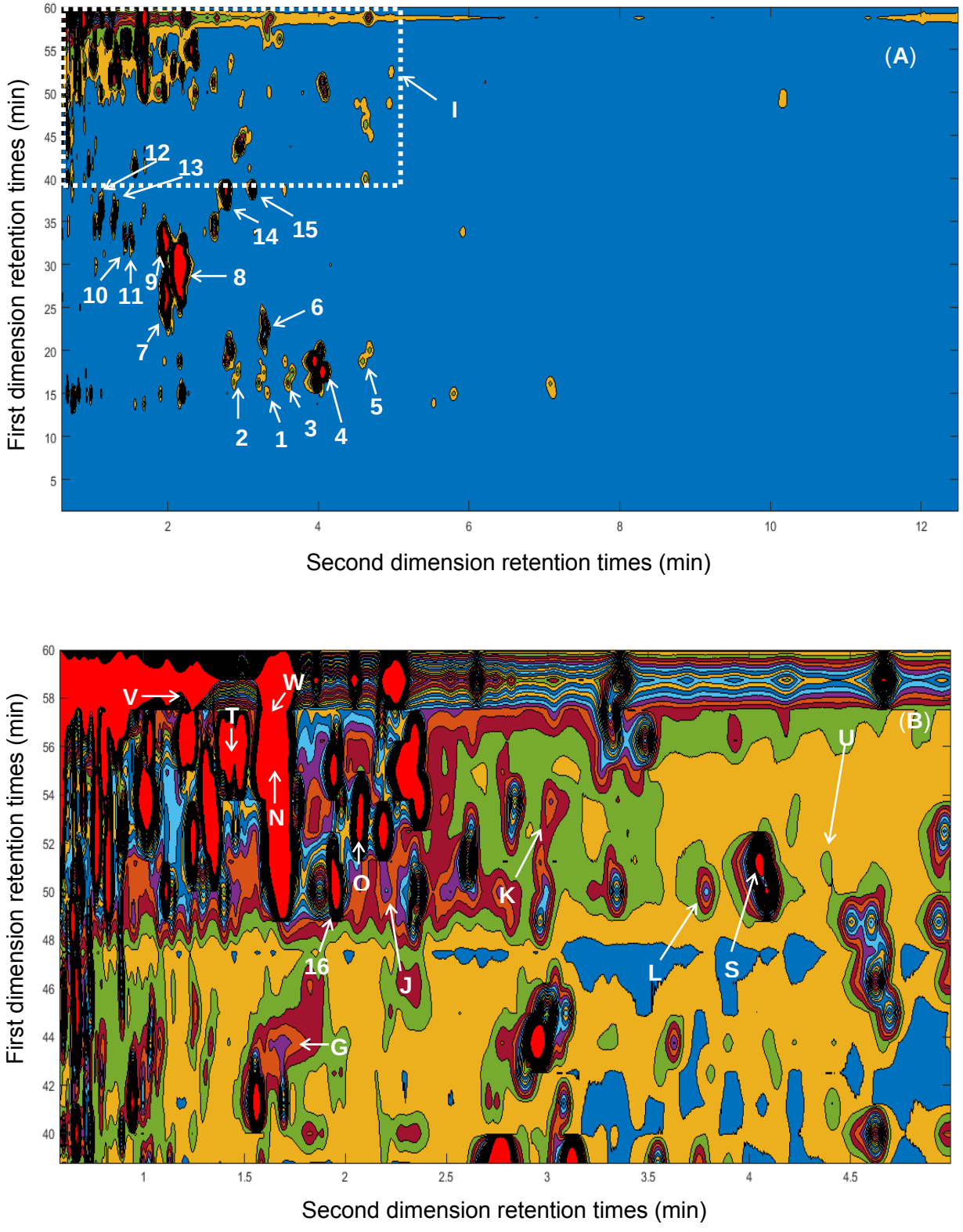


Figure 4.8 DAD contour plot representing the off-line HPCCC×RP-LC separation of a fermented rooibos extract (A), and zoomed view (corresponding to the dashed rectangular area in (A)) of the most polar compounds (B). Peak labels correspond to **Table 4.1-4.4**.

In order to determine the practical performance of the HPCCC×RP-LC methodology, some measure of the orthogonality of the system is required. Orthogonality can be determined by using discretised methods, such as information theory, or non-discretised methods such as the convex hull approach. Consensus has not been reached as to which method(s) are best suited to determine orthogonality (Schure & Davis, 2015). In this study, the orthogonality was determined using the conditional entropy (Pourhaghighi *et al.*, 2011) and convex hull (Semard *et al.*, 2010) methods. Values of 80.2% and 81.0% were obtained for orthogonality of the HPCCC×UHPLC-DAD method according to the conditional entropy and convex hull methods, respectively (a value of 100% indicates complete orthogonality, whereas 0% indicates no difference between separation mechanisms in both dimensions). Both methods confirm the high orthogonality for the 2D separation, and provide remarkably similar values. Differences between the values obtained were expected due to the fact that the two methods process data differently. The conditional entropy method was set to detect peaks with a UV-Vis absorbance of 2 mAU or higher, to prevent changes in solvents during the elution gradient being detected as peaks. The convex hull method requires manual input of ^{1}D and ^{2}D retention times for each compound at the highest concentration point. The small difference in orthogonality values determined using these two methods provides good evidence that the values are relatively accurate.

Using a value of 80%, the practical peak capacity of the separation can now be determined by taking into account both the effects of under-sampling and finite orthogonality according to **equation 9**.

This approach provides an effective practical peak capacity of 3293, and a corresponding peak capacity production rate of 3.23 min^{-1}. The performance of the 2D HPCCC×UHPLC method compares favourably to that of the off-line HILIC×RP-LC analysis of green and fermented rooibos reported by Beelders *et al.*, (2012a). The latter method had a practical peak capacity of 2095 and a peak production rate of 1.00 min^{-1}.

3.4. High Performance Countercurrent Chromatography (HPCCC)×UHPLC-MS Analysis of Green and Fermented Rooibos

In order to identify the separated phenolic compounds, the ^{2}D separation of HPCCC fractions was hyphenated to ESI-Q-TOFMS(/MS) in positive ionisation mode with the goal of gaining structural information to tentatively identify compounds. Although phenolic compounds such as *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) and ferulic acid were not detected in positive ionisation (Allwood & Goodacre, 2010), this mode was chosen as it is best used for structural elucidation of flavonoids (Cuyckens & Claeys, 2004).

The total ^{2}D MS analysis time was 17 h per sample. Single stage MS data revealed that the green rooibos sample contained fewer phenolic compounds compared to the fermented sample (indicated in **Tables 4.1-4.4**): the fermented sample contained the all the phenolic compounds present in the green sample, in addition to a number of other phenolics. The contour plots (**Figures 4.7** and **4.8**) confirm that the fermented rooibos extract has a more complex phenolic composition, although lower levels of the major phenolic compounds such as aspalathin are present. For this reason, only data obtained for the fermented sample are shown in **Tables 4.1-4.4**. In the case of well-known rooibos phenolic compounds, relative retention times, as well as UV-Vis, single stage MS and fragmentation data obtained by MS^{E} analysis were sufficient to confirm their presence (**Table 4.1**). However, for some compounds it was difficult to assign subclasses based on UV-Vis data or to obtain clear fragmentation patterns due to their low quantities. In order to obtain better quality spectra for these compounds, four HPCCC separations were performed for the fermented rooibos extract, and corresponding fractions from all runs were pooled and reconstituted to a higher concentration after evaporation of solvents. Fractions were selected for targeted MS/MS analysis of compounds where MS^{E} data did not provide conclusive assignments. The total analysis time for the selected fractions by MS/MS was 8 h.

Table 4.1 Characteristics of phenolic compounds identified in green and fermented rooibos extracts based on UV-Vis and MS^E data

^{1}D fraction number	^{2}D retention time (min)	Peak nr	λ_{max} (nm)	Accurate mass experimental ($[M+H]^+$)	Proposed molecular formula	Error (ppm)	MS^E fragments	Phenolic compound
12	3.47	**1**	223, 288, 340	465.1022	$C_{21}H_{21}O_{12}$	-2.4	303*	Hyperoside
13	3.23	**2**	226, 267, 339	433.1133	$C_{21}H_{21}O_{10}$	-0.1	313*, 283	Vitexin
13	3.44	**3**	266, 346	465.1021	$C_{21}H_{21}O_{12}$	-2.1	303*	Isoquercitrin
13	4.14	**4**	267, 344	449.1098	$C_{21}H_{21}O_{11}$	3.1	287*	Luteolin-7-*O*-glucopyranoside
13	4.64	**5**	221, 287	437.1452	$C_{21}H_{25}O_{10}$	0.9	353*, 193, 107	Nothofagin[a]
15	3.76	**6**	267, 338	433.1131	$C_{21}H_{21}O_{10}$	-0.9	313, 283*	Isovitexin
18	2.31	**7**	256, 347	449.1089	$C_{21}H_{21}O_{11}$	1.1	329*, 299	Orientin
20	2.45	**8**	239, 288	453.1405	$C_{21}H_{25}O_{11}$	1.9	193, 165*, 123	Aspalathin
24	2.32	**9**	238, 288	449.1084	$C_{21}H_{21}O_{11}$	0.0	329, 299*	Iso-orientin
26	1.72	**10**	235, 289	451.1231	$C_{21}H_{23}O_{11}$	-2.0	301, 195, 165*	(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]
26	1.83	**11**	239, 290	451.1233	$C_{21}H_{23}O_{11}$	-1.6	301, 195, 165*	(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside[a]
28	1.29	**12**	237,289	451.1234	$C_{21}H_{23}O_{11}$	-0.6	301, 195*, 165	(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]
28	1.49	**13**	235, 290	451.1229	$C_{21}H_{23}O_{11}$	-2.4	301, 195*, 165	(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside[a]
31	3.42	**14**	284, 346	611.1596	$C_{27}H_{31}O_{16}$	-2.9	465, 303*	Quercetin-3-*O*-robinobioside[a]
32	3.77	**15**	284, 346	611.1607	$C_{27}H_{31}O_{16}$	-0.5	465, 303*	Rutin
40	1.89	**16**	226, 278	327.1086	$C_{15}H_{19}O_8$	1.8	165*, 119	*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)[a]

* Fragment with maximum intensity. *Abbreviations:* ^{1}D, first dimension; ^{2}D, second dimension.

Table 4.2 Characteristics of dihydrochalcones detected in fermented rooibos extract based on UV-Vis, MS and MS/MS data

^{1}D fraction number	^{2}D retention time (min)	Peak nr	λ_{max} (nm)	Accurate mass experimental ($[M+H]^{+}$)	Proposed molecular formula	Error (ppm)	MS/MS fragment ions	Phenolic compound
13	6.4	**B**	238, 285	741.2010	$C_{36}H_{37}O_{17}$	-2.8	553, 451, 421, 329, 299, 289, 247*, 193, 165, 135,123	Aspalathin derivative#
41	5.36	**I**	231, 285	599.1967	$C_{27}H_{35}O_{15}$	-1.5	509, 491, 479, 461, 449, 431, 419, 389, 359, 329, 299, 151, 149, 107*	Phloretin-3',5'-di-*C*-glucoside
44	1.69	**M**	233, 283	583.1651	$C_{26}H_{31}O_{15}$	-2.1	493, 475, 463, 445, 433, 415, 385, 373, 355, 343, 283, 193, 165, 123*	Aspalathin derivative#
44	2.12	**O**	279	597.1808	$C_{27}H_{33}O_{15}$	-1.8	507, 477, 447, 301, 297, 277, 259, 247, 165, 123*	Aspalathin derivative
44	2.23	**P**	224, 271	901.2392	$C_{42}H_{45}O_{22}$	-1.1	811, 781, 751, 721, 691, 661, 631, 601, 339*, 163	Aspalathin dimer#
44	2.54	**Q**	233, 286	901.2413	$C_{42}H_{45}O_{22}$	1.2	811, 781, 751, 721, 691, 661, 631, 601, 339*, 163	Aspalathin dimer#
44	3.56	**R**	230, 281	901.2420	$C_{42}H_{45}O_{22}$	2.0	811, 781, 751, 721, 691, 661, 631, 601, 339*, 163	Aspalathin dimer#
45	4.35	**S**	228, 278	583.1657	$C_{26}H_{31}O_{15}$	-1.0	523, 493, 475, 463, 445, 433, 415, 385, 373, 355, 343, 325, 193, 165, 123*	Aspalathin derivative
45	1.91	**T**	229, 281	615.1907	$C_{27}H_{35}O_{16}$	-2.0	495, 465, 435, 363, 333, 303, 193, 165, 123*	3-hydroxyphloretin-3',5'-di-*C*-hexoside
45	4.9	**U**	233, 284	873.2458	$C_{41}H_{45}O_{21}$	0.6	813, 783, 735, 723, 705, 675, 645, 615, 567*, 507, 373, 343, 313, 283, 253, 223, 193, 165, 123	Aspalathin derivative

* Fragment with maximum intensity; # Peaks not detected in green rooibos extracts. *Abbreviations:* ^{1}D, first dimension; ^{2}D, second dimension.

Table 4.3 Characteristics of flavanones detected in fermented rooibos extract based on UV-Vis, MS and MS/MS data

^{1}D fraction number	^{2}D retention time (min)	Peak nr	λmax (nm)	Accurate mass experimental ([M+H]+)	Proposed molecular formula	Error (ppm)	MS/MS fragment ions	Phenolic compound
28	8.05	**D**	242, 284	611.1977	$C_{28}H_{35}O_{15}$	0.2	303*, 195, 177, 153	Hesperidin[#]
33	2.15	**G**	230, 282	447.1279	$C_{22}H_{23}O_{10}$	-2.7	285*, 270, 242,153, 123, 96	Flavanone-*O*-hexoside
35	5.78	**H**	245, 285	533.1277	$C_{25}H_{25}O_{13}$	-3.4	455, 371, 285*, 270, 153, 91	Flavanone-*O*-malonylhexoside[#]

* Fragment with maximum intensity; [#] Peaks not detected in green rooibos extracts. *Abbreviations:* ^{1}D, first dimension; ^{2}D, second dimension.

Table 4.4 Characteristics of flavones detected in fermented rooibos extract based on UV-Vis, MS and MS/MS data

^{1}D fraction number	^{2}D retention time (min)	Peak nr	λ_{max} (nm)	Accurate mass experimental ($[M+H]^+$)	Proposed molecular formula	Error (ppm)	MS/MS fragment ions	Phenolic compound
12	8.5	**A**	267, 338	463.1239	$C_{22}H_{23}O_{11}$	-0.2	301*, 286, 258, 229, 153	Chrysoeriol-*O*-hexoside[#]
19	9.65	**C**	251, 346	535.1081	$C_{24}H_{23}O_{14}$	-1.3	449, 287*, 153, 137	Luteolin-*O*-malonylhexoside[#]
28	8.35	**E**	244, 268, 345	609.1821	$C_{28}H_{33}O_{15}$	0.3	463, 301*, 286, 258, 177, 117	Chrysoeriol-*O*-neohesperidoside[#]
31	4.72	**F**	265, 348	595.1655	$C_{27}H_{31}O_{15}$	-1.3	449, 287*, 153	Scolymoside[#]
43	2.26	**J**	231, 271, 332	565.1567	$C_{26}H_{29}O_{14}$	1.8	415, 391*, 385, 379, 355, 325, 295, 207, 203, 163, 121	Apigenin-*C*-hexoside-*C*-pentoside
43	2.64	**K**	227, 270, 335	565.1546	$C_{26}H_{29}O_{14}$	-1.9	415, 391, 385, 379, 355, 325, 295*, 207, 203, 163, 121	Apigenin-*C*-hexoside-*C*-pentoside
43	3.71	**L**	228, 270, 332	565.1547	$C_{26}H_{29}O_{14}$	-1.8	415, 391, 385, 379*, 355, 325, 295, 207, 203, 163, 121	Apigenin-*C*-hexoside-*C*-pentoside
44	1.85	**N**	271, 344	581.1511	$C_{26}H_{29}O_{15}$	0.9	491, 461, 455, 431, 425, 407, 395*, 371, 365, 341, 311, 203, 137	Luteolin-*C*-hexoside-*C*-deoxyhexoside
45	1.09	**V**	230, 271, 343	611.1612	$C_{27}H_{31}O_{16}$	0.0	491, 473, 395, 365, 353*, 329, 299, 134	Luteolin-di-*C*-hexoside
45	1.61	**W**	230, 270, 340	595.1650	$C_{27}H_{31}O_{15}$	-2.2	517, 505, 487, 475, 457, 427, 397, 379, 367, 355, 337, 325*, 295, 163, 121	Vicenin 2

* Fragment with maximum intensity; [#] Peaks not detected in green rooibos extracts. *Abbreviations:* ^{1}D, first dimension; ^{2}D, second dimension.

As mentioned in the literature chapter, rooibos phenolics and their associated isomers have distinct fragmentation patterns which can be used for their tentative identification (Abad-Garcia *et al.*, 2008). It should be noted though that the compound assignments derived from UV-Vis, single stage MS and MS/MS characteristics remain tentative, as molecular isomers for example cannot be unambiguously assigned in the absence of standards.

In the following discussion, the nomenclature used to describe the fragmentation will be in accordance with Abad-Garcia *et al.*, (2008), Abad-Garcia *et al.*, (2009) and Cao *et al.*, (2014), which is represented in **Figure 4.9** for the dihydrochalcones. Distinguishing between different *C*-glycosides, due to the complexity of the associated mass spectra, is challenging. For these compounds, fragmentation typically involves cross-ring cleavages of sugar moieties. Product ions denoted $[^{0,3}X]^+$, $[^{0,2}X]^+$, and $[^{0,1}X]^+$ correspond to mass losses of 90, 120 and 150 amu for hexoses, and losses of 60, 90 and 120 for pentoses (Cao *et al.*, 2014). Fragmentation of *C*-hexosides will therefore result in $[M-120/90]^{+/-}$ ions, while *C*-pentosides produce $[M-134/90/60]^{+/-}$ and *C*-deoxyhexosides $[M-104/74]^{+/-}$ ions, respectively. These fragmentation pathways are also commonly associated with the loss of water $[M-18]^{+/-}$ (Farag *et al.*, 2016). In contrast to the complex *C*-glycoside spectra, *O*-glycosides have less complex MS spectra and fragmentation patterns. Since the *O*-glycosidic bonds are relatively weak and are cleaved under low collision energy conditions, neutral loss of a sugar moiety will be diagnostic.

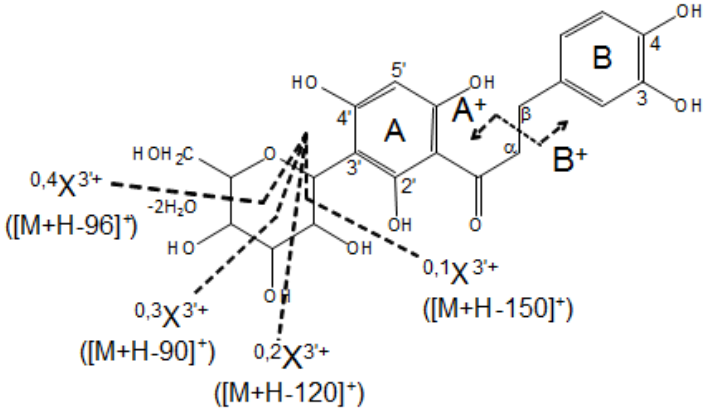


Figure 4.9 The general MS fragmentation pattern for dihydrochalcone *C*-glycosides using aspalathin as example (Beelders *et al.*, 2012b).

Table 4.2 lists the compounds tentatively identified as dihydrochalcones. Beelders *et al.*, (2012c) noted fragment ions at *m/z* 123.0446 ($C_7H_7O_2$) (B^+) and *m/z* 165.0552 ($C_9H_9O_3$)

in the high collision energy MS/MS spectrum of aspalathin in positive mode. These ions were also present in the MS/MS spectra of peaks **B**, **M**, **O**, **S**, **T** and **U**, which allowed their assignment as aspalathin derivatives. Peak **B** showed fragment ions corresponding to multiple losses of 90, 120 and 134 amu, but no losses of 104 amu, indicating the presence of two *C*-linked hexosides (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009). This is also true for compounds differing from known compounds by an additional OH, double bond etc. Unfortunately, this compound could not be further identified based on the available data.

The MS/MS spectra of compounds **I**, **M**, **O**, **S** and **T** showed multiple mass losses of 90, 120, 150 amu, as well as associated water losses, which indicated the presence of two C-linked hexosides. Peak **I** was identified as phloretin-3',5'-di-*C*-glucoside using an authentic reference standard isolated from *Cyclopia subternata*, while peak **T** was tentatively identified as 3-hydroxyphloretin-3',5'-di-*C*-hexoside. The latter compound was first identified in rooibos (Beelders *et al.*, 2012c) and later also in several *Cyclopia* spp (honeybush) (Beelders *et al.*, 2012a; De Beer *et al.*, 2012; Schulze *et al.*, 2014; Schulze *et al.*, 2015). Peaks **M** and **S** are likely isomers as both have the same pseudomolecular ion, *m/z* 583.1663 ($C_{26}H_{31}O_{15}$) as well as similar MS/MS fragments and UV-Vis spectra. According to experimental accurate mass data, these compounds differ from phloretin-3',5'-di-*C*-glucoside by a CH_4 functional group, but further assignment of the structures was not possible. Peaks **P**, **Q**, **R** and **U**, were tentatively identified as dimers of aspalathin as reported by Krafczyk *et al.*, (2009) and Heinrich *et al.*, (2012). Compounds **P**, **Q** and **R** are isomers of each other as they have similar MS/MS spectra and molecular ions *m/z* 901.2402 ($C_{42}H_{45}O_{22}$). **Figure 4.10** presents the MS/MS spectrum of peak **Q**, which illustrates the fragmentation of these dimeric species. None of the compounds **B**, **I**, **M**, **O**, **S**, **T** and **U** have previously been reported in rooibos extracts.

Table 4.3 lists three tentatively identified flavanones. Peak **D** was identified as hesperidin based on an authentic reference standard. Compounds **G** and **H** were tentatively identified as flavanones based first of all on their UV-Vis spectra. These compounds could potentially be dihydrochalcones, but this seems unlikely due to the lack of the characteristic aspalathin or phloretin fragments. Peak **G** was tentatively identified as a flavanone-*O*-hexoside based on a fragment ion corresponding to the loss of a hexoside ($[M+H-162]^+$) at *m/z* 285.0763 ($C_{16}H_{13}O_5$). Peak **H** was tentatively identified as a flavanone-*O*-malonylhexoside based on fragments detected at *m/z* 371.0767 ($C_{19}H_{15}O_8$) and 285.0763 ($C_{16}H_{13}O_5$), which correspond to the loss of hexoside ($[M+H-162]^+$) and malonylhexoside groups ($[M+H-162-86]^+$), respectively (Cuyckens & Claeys, 2004). The base peak ion at *m/z* 285.0753 ($C_{16}H_{13}O_5$), corresponding to the aglycone ion for both compounds, does however not conform to any known flavanone aglycone. Therefore, without nuclear magnetic resonance (NMR) analyses it is not be possible to unambiguously determine the structure of

the two compounds in question. This is the first time compounds with the mass spectral properties of compounds **D**, **G** and **H** are reported in rooibos extracts.

Table 4.4 contains a list of the flavones detected in the fermented rooibos extract. Peaks **A** and **E** was tentatively identified as chrysoeriol-*O*-glycosides based on a base peak ion at *m/z* 301.0712 ($C_{16}H_{13}O_6$) combined with the fact that chrysoeriol – unlike diosmetin - have previously been found in rooibos (Beelders *et al.*, 2012c). Peak **A** was tentatively identified as a chrysoeriol-*O*-hexoside based on the neutral loss of 162 amu. Peak **E** was identified as a chrysoeriol-*O*-neohesperidoside based on fragment ions at *m/z* 463.1240 ($C_{22}H_{23}O_{11}$) ($[M+H-146]^+$) and 301.0712 ($C_{16}H_{13}O_6$) ($[M+H-146-162]^+$) in accordance with Abad-García *et al.*, (2009).

Peaks **C** and **F** were identified as luteolin-*O*-glycosides based on a base peak ion in the MS/MS spectrum at *m/z* 287.0558 ($C_{15}H_{11}O_6$). Peak **C** was tentatively identified as luteolin-*O*-malonylhexoside as described for peak **H** (Cuyckens & Claeys, 2004). Peak **F** was identified as scolymoside based on comparison with an authentic reference standard. This compound has previously been identified in several *Cyclopia* spp (Schulze *et al.*, 2014; Schulze *et al.*, 2015).

Peaks **J**, **K** and **L** were tentatively identified as apigenin-*C*-hexoside-*C*-pentoside isomers as the UV-Vis, single stage MS and MS/MS spectra were in accordance with those obtained by Benayad *et al.*, (2014) for vicenin 1 and 3. It is proposed that compounds **J** and **K** contain 8-*C*-pentoside and 6-*C*-hexoside moieties, respectively, while peak **L** contains 8-*C*-hexoside and 6-*C*-pentoside moieties, based on the fact that 8-*C*-pentosides elute earlier than 8-*C*-hexosides in reversed phase mode (Benayad *et al.*, 2014). The fragment ions *m/z* 415.0877 $[M+H-150]^+$, *m/z* 325.0712 $[M+H-150-90]^+$ and *m/z* 295.0606 $[M+H-150-120]^+$, confirm the presence of both *C*-hexoside and *C*-pentoside moieties (Cuyckens & Claeys, 2004; Abad-Garcia *et al.*, 2009). Peak **N** was tentatively identified as luteolin-*C*-hexoside-*C*-deoxyhexoside based on the fragment ions *m/z* 491.1037 $[M+H-90]^+$, *m/z* 461.0931 $[M+H-120]^+$, *m/z* 431.0673 $[M+H-150]^+$, *m/z* 455.0919 $[M+H-90-2H_2O]^+$, *m/z* 425.0873 $[M+H-120-2H_2O]^+$ and *m/z* 395.0767 $[M+H-150-2H_2O]^+$, suggesting the presence of *C*-hexoside and *C*-deoxyhexoside groups (Farag *et al.*, 2016). Peak **V** and **W** displayed multiple neutral losses of 90, 120 and 150 characteristic of a luteolin-di-*C*-hexoside, as observed for a vicenin-2 authentic reference standard (Cuyckens & Claeys, 2004). Peak **W** was indeed identified as vicenin-2, previously identified in rooibos by Iswaldi *et al.*, (2011) and Beelders *et al.*, (2012c), based on its retention time compared to that of the standard. Peak **A**, **C**, **E**, **F**, **J**, **K**, **L** and **N** have not previously been detected in rooibos extracts.

Using RP-LC, highly polar compounds are relatively weakly retained, and therefore hard to identify. In the HPCCC×RP-LC separation, highly polar compounds eluted in the

later NP-HPCCC fractions (41 – 45) and early in the RP-UHPLC separation; most of these compounds comprised di-*C*-glycosidic molecules (Cuyckens & Claeys, 2004). In contrast, the non-polar fractions (12-35), which eluted early in NP-HPCCC and late in RP-UHPLC, contained the majority of the mono- and di-*O*-glycosides found in rooibos (Cuyckens & Claeys, 2004).

The majority of the compounds separated and tentatively identified for the first time were flavonoid-(di)*C*-glycosides. This makes identification based solely on MS/MS spectra challenging, due to complex nature of high collision energy mass spectra for such compounds, coupled to the number of possible isomers. NMR would be required for unambiguous identification of rooibos *C*-glycosides. For NMR analysis, the compound in question must be isolated and purified. This might be achieved by upscaling both CCC and RP-LC separations, as most of the compounds were well separated following the two-dimensional separation (with the exception of fractions 44 and 45, where a few compounds co-eluted) (Crockford *et al.*, 2006; Bertrand *et al.*, 2016). The presence of phenolic acids must also be considered in such an endeavour, although their levels are generally quite low.

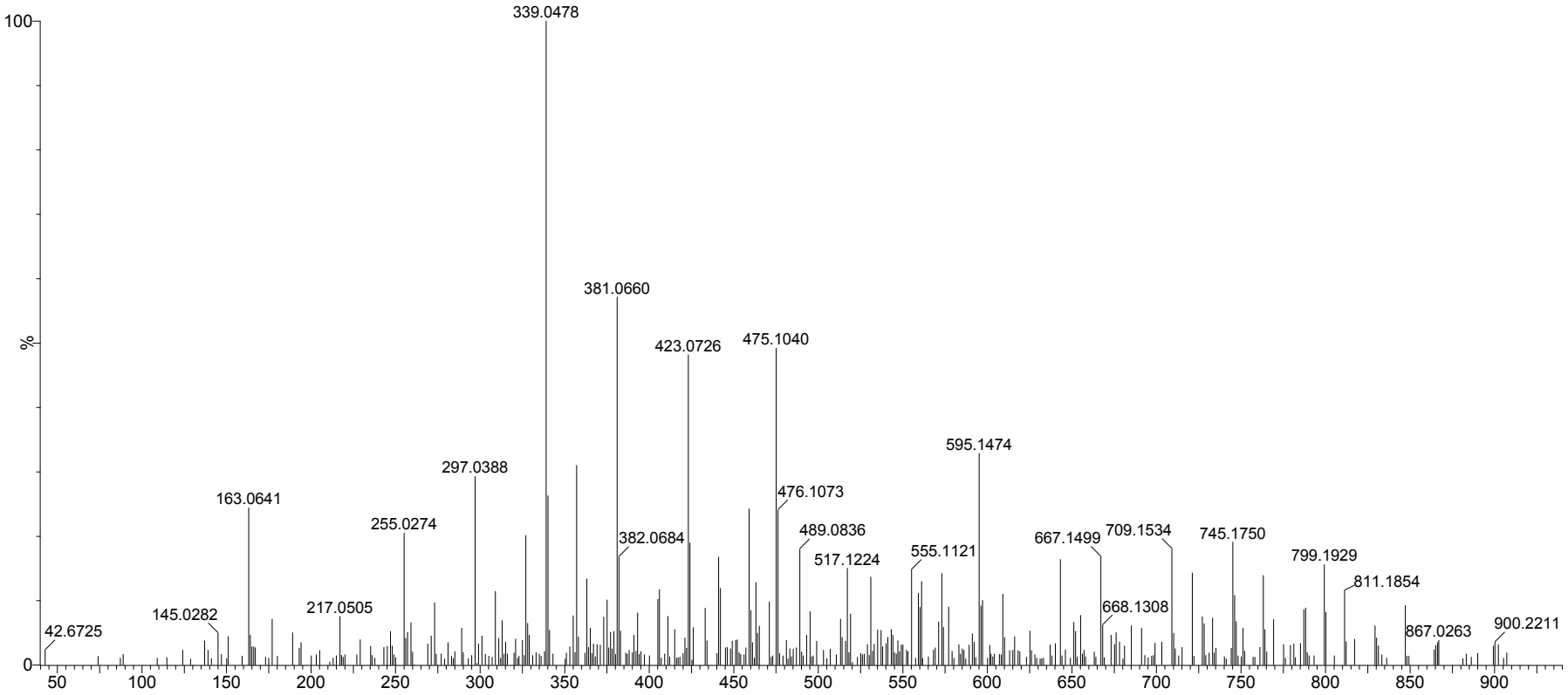


Figure 4.10 MS/MS spectrum of peak **Q** tentatively identified as an aspalathin dimer.

4. Conclusions

The off-line, comprehensive combination of NP-HPCCC (in the ^{1}D) and RP-UHPLC (in the ^{2}D) separations provided excellent separation of rooibos phenolic compounds with a high degree of orthogonality (~80%) in a total analysis time of 17 h. The large polarity range of the HPCCC gradient separation was ideal for the ^{1}D separation, since this allowed the phenolic compounds to be spread across the retention space. The higher sample loading capacity of HPCCC was also beneficial in the off-line separation, as phenolic compounds present in relatively low quantities could be detected following evaporation of the ^{1}D mobile phase. On the other hand, the high efficiency associated with UHPLC analysis was ideal for the ^{2}D as this allowed baseline separation for the majority of the rapidly eluting polar compounds. The results point to the highly complex nature of rooibos phenolic fractions. A total of 39 compounds were separated and tentatively identified. Of these, 16 were identified and quantified in the previous chapter by means of one-dimensional UHPLC-DAD-MS, whereas 23 additional compounds were detected using HPCCC×RP-LC, 18 of which are reported here in rooibos extracts for the first time. Three compounds previously reported in *Cyclopia* spp (honeybush) were identified using authentic reference standards, namely the dihydrochalcone phloretin-3',5'-di-*C*-glucoside, the flavanone hesperidin and the flavone scolymoside. Further analyses using negative mode ionisation could result in additional compounds being identified. The information obtained further enhances our knowledge on rooibos phenolic composition, which can be critical for understanding its bioactivity potential.

References

Abad-Garcia, B., Berrueta, L. A., Garmon-Lobato, S., Gallo, B. & Vicente, F. (2009). A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. *Journal of Chromatography A,* **1216**, 5398-5415.

Abad-Garcia, B., Garmon-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2008). New features on the fragmentation and differentiation of *C*-glycosidic flavone isomers by positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry,* **22**, 1834-1842.

Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2009). Practical guidelines for characterization of *O*-diglycosyl flavonoid isomers by triple quadrupole MS and their applications for identification of some fruit juices flavonoids. *Journal of Mass Spectrometry,* **44**, 1017-1025.

Allwood, J. W. & Goodacre, R. (2010). An introduction to liquid chromatography-mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochemical Analysis,* **21**, 33-47.

Beelders, T., Kalili, K. M., Joubert, E., De Beer, D. & De Villiers, A. (2012a). Comprehensive two-dimensional liquid chromatographic analysis of rooibos (*Aspalathus linearis*) phenolics. *Journal of Separation Science,* **35**, 1808-1820.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012b). Erratum to "Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation" [J. Chromatogr. A 1219 (2012) 128–139]. *Journal of Chromatography A,* **1241**, 128.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012c). Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation. *Journal of Chromatography A,* **1219**, 128-139.

Benayad, Z., Gomez-Cordoves, C. & Es-Safi, N. E. (2014). Characterization of flavonoid glycosides from fenugreek (*Trigonella foenum-graecum*) crude seeds by HPLC-DAD-ESI/MS analysis. *International Journal of Molecular Sciences,* **15**, 20668-20685.

Berthod, A., Maryutina, T., Spivakov, B., Shpigun, O. & Sutherland, I. A. (2009). Countercurrent chromatography in analytical chemistry (IUPAC technical report). *Pure and Applied Chemistry,* **81**, 355-387.

Bertrand, S., Azzollini, A., Nievergelt, A., Boccard, J., Rudaz, S., Cuendet, M. & Wolfender, J. L. (2016). Statistical correlations between HPLC activity-based profiling results and NMR/MS microfraction data to deconvolute bioactive compounds in mixtures. *Molecules,* **21**, 259-271.

Cao, J., Yin, C., Qin, Y., Cheng, Z. & Chen, D. (2014). Approach to the study of flavone di-*C*-glycosides by high performance liquid chromatography-tandem ion trap mass spectrometry and its application to characterization of flavonoid composition in *Viola yedoensis*. *Journal of Mass Spectrometry,* **49**, 1010-1024.

Cardozo Junior, E. L. & Morand, C. (2016). Interest of mate (*Ilex paraguariensis* A. St.-Hil.) as a new natural functional food to preserve human cardiovascular health – A review. *Journal of Functional Foods,* **21**, 440-454.

Chen, W. B., Li, S. Q., Chen, L. J., Fang, M. J., Chen, Q. C., Wu, Z., Wu, Y. L. & Qiu, Y. K. (2015). Online polar two phase countercurrent chromatography x high performance liquid chromatography for preparative isolation of polar polyphenols from tea extract in a single step. *Journal of Chromatography B,* **997**, 179-186.

Costa, F. & Leitao, G. G. (2011). Evaluation of different solvent systems for the isolation of *Sparattosperma leucanthum* flavonoids by counter-current chromatography. *Journal of Chromatography A,* **1218**, 6200-6205.

Costa, F. & Leitão, G. G. (2010). Strategies of solvent system selection for the isolation of flavonoids by countercurrent chromatography. *Journal of Separation Science,* **33**, 336-347.

Crockford, D. J., Holmes, E., Lindon, J. C., Plumb, R. S., Zirah, S., Bruce, S. J., Rainville, P., Stumpf, C. L. & Nicholson, J. K. (2006). Statistical heterospectroscopy, an approach to the integrated analysis of NMR and UPLC-MS data sets: Application in metabonomic toxicology studies. *Analytical Chemistry,* **76**, 363-371.

Cuyckens, F. & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry,* **39**, 1-15.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I. & Heinrich, M. (2014). *Hibiscus sabdariffa* L. - a phytochemical and pharmacological review. *Food Chemistry,* **165**, 424-443.

De Beer, D., Malherbe, C. J., Beelders, T., Willenburg, E. L., Brand, D. J. & Joubert, E. (2015). Isolation of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) using high-performance countercurrent chromatography: Sample loading and compound stability considerations. *Journal of Chromatography A,* **1381**, 29-36.

De Beer, D., Schulze, A., Joubert, E., De Villiers, A., Malherbe, C. & Stander, M. (2012). Food Ingredient extracts of *Cyclopia subternata* (Honeybush): variation in phenolic composition and antioxidant capacity. *Molecules,* **17**, 14602-14624.

De Villiers, A., Venter, P. & Pasch, H. (2016). Recent advances and trends in the liquid-chromatography-mass spectrometry analysis of flavonoids. *Journal of Chromatography A,* **1430**, 16-78.

Farag, M. A., Otify, A., Porzel, A., Michel, C. G., Elsayed, A. & Wessjohann, L. A. (2016). Comparative metabolite profiling and fingerprinting of genus *Passiflora* leaves using a multiplex approach of UPLC-MS and NMR analyzed by chemometric tools. *Analytical and Bioanalytical Chemistry,* **408**, 3125-3143.

Friesen, B. & Pauli, G. F. (2005). G.U.E.S.S.—A generally useful estimate of solvent systems for CCC. *Journal of Liquid Chromatography & Related Technologies,* **28**, 2777-2806.

Garrard, I. J., Janaway, L. & Fisher, D. (2007). Minimising solvent usage in high speed, high loading, and high resolution isocratic dynamic extraction. *Journal of Liquid Chromatography & Related Technologies,* **30**, 151-163.

Giddings, J. C. (1967). Maximum number of components resolvable by gel filtration and other elution chromatographic methods. *Analytical Chemistry,* **39**, 1027-1028.

Gilar, M., Olivova, P., Daly, A. E. & Gebler, J. C. (2005). Orthogonality of separation in two-dimensional liquid chromatography. *Analytical Chemistry,* **77**, 6426-6434.

González-Ruiz, V., Olives, A. I. & Martín, M. A. (2015). Core-shell particles lead the way to renewing high-performance liquid chromatography. *Trends in Analytical Chemistry,* **64**, 17-28.

Heinrich, T., Willenberg, I. & Glomb, M. A. (2012). Chemistry of color formation during rooibos fermentation. *Journal of Agricultural and Food Chemistry,* **60**, 5221-5228.

Hopmann, E., Arlt, W. & Minceva, M. (2011). Solvent system selection in counter-current chromatography using conductor-like screening model for real solvents. *Journal of Chromatography A,* **1218**, 242-250.

Iswaldi, I., Arraez-Roman, D., Rodriguez-Medina, I., Beltran-Debon, R., Joven, J., Segura-Carretero, A. & Fernandez-Gutierrez, A. (2011). Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Analytical and Bioanalytical Chemistry,* **400**, 3643-3654.

Joubert, E. (1996). HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. *Food Chemistry,* **55**, 403-411.

Kalili, K. M. & De Villiers, A. (2013). Systematic optimisation and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography x reversed phase liquid chromatographic analysis of procyanidins, part I: Theoretical considerations. *Journal of Chromatography A,* **1289**, 58-68.

Kamakura, R., Son, M. J., De Beer, D., Joubert, E., Miura, Y. & Yagasaki, K. (2015). Antidiabetic effect of green rooibos (*Aspalathus linearis*) extract in cultured cells and type 2 diabetic model KK-A(y) mice. *Cytotechnology,* **67**, 699-710.

Krafczyk, N., Heinrich, T., Porzel, A. & Glomb, M. A. (2009). Oxidation of the dihydrochalcone aspalathin leads to dimerization. *Journal of Agricultural and Food Chemistry,* **57**, 6838-6843.

Ku, S. K., Kwak, S., Kim, Y. & Bae, J. S. (2015a). Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibits high glucose-induced inflammation *in vitro* and *in vivo*. *Inflammation,* **38**, 445-455.

Ku, S. K., Lee, W., Kang, M. & Bae, J. S. (2015b). Antithrombotic activities of aspalathin and nothofagin via inhibiting platelet aggregation and FIIa/FXa. *Archives of Pharmacal Research,* **38**, 1080-1089.

Kwak, S., Han, M. S. & Bae, J. S. (2015). Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibit endothelial protein C receptor shedding *in vitro* and *in vivo*. *Fitoterapia,* **100**, 179-186.

Leitao, G. G. & Costa, F. D. (2015). Gradient elution in countercurrent chromatography. *Planta Medica,* **81**, 1592-1596.

Lerotholi, L., Chaudhary, S. K., Combrinck, S. & Viljoen, A. (2016). Bush tea (*Athrixia phylicoides*): A review of the traditional uses, bioactivity and phytochemistry. *South African Journal of Botany,* DOI:10.1016/j.sajb.2016.06.005.

Liu, Y., Friesen, J. B., McAlpine, J. B. & Pauli, G. F. (2015). Solvent system selection strategies in countercurrent separation. *Planta Medica,* **81**, 1582-1591.

Mekaoui, N., Faure, K. & Berthod, A. (2012). Advances in countercurrent chromatography for protein separations. *Bioanalysis,* **7**, 833-844.

Michel, T., Destandau, E. & Elfakir, C. (2011). On-line hyphenation of centrifugal partition chromatography and high pressure liquid chromatography for the fractionation of flavonoids from *Hippophae rhamnoides L.* berries. *Journal of Chromatography A,* **1218**, 6173-6178.

Muller, C. J., Joubert, E., De Beer, D., Sanderson, M., Malherbe, C. J., Fey, S. J. & Louw, J. (2012). Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. *Phytomedicine,* **20**, 32-39.

Muller, C. J., Malherbe, C. J., Chellan, N., Yagasaki, K., Miura, Y. & Joubert, E. (2016). Potential of rooibos, its major *C*-glucosyl flavonoids and *Z*-2-(beta-D-glucopyranoloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. *Critical Reviews in Food Science and Nutrition,* DOI: 10.1080/10408398.2016.1157568.

Neue, U. D. (2005). Theory of peak capacity in gradient elution. *Journal of Chromatography A,* **1079**, 153-161.

Oh, J., Jo, H., Cho, A. R., Kim, S.-J. & Han, J. (2013). Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control,* **31**, 403-409.

Pourhaghighi, M. R., Karzand, M. & Girault, H. H. (2011). Orthogonality of two-dimensional separations based on conditional entropy. *Analytical Chemistry,* **83**, 7676-7681.

Schulze, A. E., Beelders, T., Koch, I. S., Erasmus, L. M., De Beer, D. & Joubert, E. (2015). Honeybush herbal teas (*Cyclopia* spp.) contribute to high levels of dietary exposure to xanthones, benzophenones, dihydrochalcones and other bioactive phenolics. *Journal of Food Composition and Analysis,* **44**, 139-148.

Schulze, A. E., De Beer, D., De Villiers, A., Manley, M. & Joubert, E. (2014). Chemometric analysis of chromatographic fingerprints shows potential of *Cyclopia maculata* (Andrews) Kies for production of standardized extracts with high xanthone content. *Journal of Agricultural and Food Chemistry,* **62**, 10542-10551.

Schure, M. R. & Davis, J. M. (2015). Orthogonal separations: Comparison of orthogonality metrics by statistical analysis. *Journal of Chromatography A,* **1414**, 60-76.

Semard, G., Peulon-Agasse, V., Bruchet, A., Bouillon, J. P. & Cardinael, P. (2010). Convex hull: a new method to determine the separation space used and to optimize operating conditions for comprehensive two-dimensional gas chromatography. *Journal of Chromatography A,* **1217**, 5449-5454.

Sissing, L., Marnewick, J., De Kock, M., Swanevelder, S., Joubert, E. & Gelderblom, W. (2011). Modulating effects of rooibos and honeybush herbal teas on the development of esophageal papillomas in rats. *Nutrition and Cancer,* **63**, 600-610.

Van der Merwe, J. D., Joubert, E., Richards, E. S., Manley, M., Snijman, P. W., Marnewick, J. L. & Gelderblom, W. C. (2006). A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutation Research,* **611**, 42-53.

Chapter 5

General discussion, recommendations and conclusions

The consumption of *Aspalathus linearis* (rooibos) is associated with beneficial health properties (Joubert & De Beer, 2011), which have been linked to the phenolic content of rooibos (Kawano *et al.*, 2009; Van der Merwe *et al.*, 2010). These beneficial properties include; anti-oxidant potential, anti-mutagenic, anti-microbial and anti-diabetic (Marnewick *et al.*, 2005; Van der Merwe *et al.*, 2006; Marnewick *et al.*, 2009; Hübsch *et al.*, 2014; Muller *et al.*, 2016). The majority of the beneficial properties have to date only been observed by *in vitro* conditions, but *in vivo* experiments are becoming more popular as *in vivo* techniques improve and the popularity of rooibos as a herbal tea increases under the general population (Kamakura *et al.*, 2015; Ku *et al.*, 2015; Lee & Bae, 2015). Various phenolic compounds present in rooibos have not been identified due to lack of sufficient separation or complete co-elution. Constant improvement of separation techniques has led to the development of powerful multidimensional separation techniques (Shalliker, 2009; Kalili & De Villiers, 2010; Beelders *et al.*, 2012a) which, if applied, might allow improved separation of phenolic compounds and subsequent identification of the newly separated compounds.

The first objective of this study was to develop a quantitative reversed phase (RP) high performance liquid chromatography (HPLC) diode array detector (DAD) method to enable quantification of the major phenolic compounds, including specific known aspalathin oxidation products, namely eriodictyol-glucopyranosides. An extraction experiment was also performed using ethanol and acetonitrile solutions to determine the best solvent for maximum extraction of rooibos phenolic compounds from the plant material. The effect of fermentation on the phenolic composition of rooibos plant material and hot water extracts could then be studied in more detail than was previously possible. The second main objective was to design an off-line comprehensive two-dimensional (2D) method combining high performance countercurrent chromatography (HPCCC) in the first dimension (^{1}D) with ultra-high performance liquid chromatography (UHPLC)-DAD and UHPLC coupled to mass spectrometry (UHPLC-MS) in the second dimension (^{2}D). The aim was to gain detailed qualitative insight into the phenolic compounds present in green and fermented rooibos, especially for the more polar compounds which are not well resolved by one dimensional HPLC.

An optimised RP-HPLC-DAD method was designed based on the method used by Beelders *et al.*, (2012b) to separate and quantify the flavanones (*R*)- and (*S*)-eriodictyol-6-*C*-glucopyranoside and -8-*C*-glucopyranoside, as well as the dihydrochalcone aspalathin and the flavones iso-orientin and orientin. The goal was to retain as far as possible the phenolic compound separation obtained by Beelders *et al.*, (2012b), while at the same time obtaining accurate quantitative data for the abovementioned flavanones. The developed method could then be used to determine the effect of fermentation on the target phenolic compounds present in rooibos. The final method proved suitable for the separation and quantification of

all the target flavonoids, as well as nothofagin, *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG), quercetin-3-*O*-robinobioside, vitexin, iso-vitexin, isoquercitrin, hyperoside, rutin and luteolin-7-*O*-glucopyranoside. As the separation reported by Beelders *et al.*, (2012b) could not be replicated on three newly purchased columns of the same specifications as used in the original method (ZORBAX SB C_{18}, 100 x 4.6 mm i.d., 1.8 µm), a new method was developed on a conventional HPLC instrument and a Poroshell SB C_{18} (150 x 4.6 mm i.d., 2.7 µm) column. This core-shell column provides similar performance to fully porous phases of smaller particle size, due to a small particle size distribution and improved packing efficiency (Hayes *et al.*, 2014). This allowed the use of a large particle diameter of 2.7 µm, as opposed to the 1.8 µm fully porous phase used in the original method, which resulted in reduced back-pressures. As a consequence, a 150 mm Poroshell column could be used, resulting in improved separation of critical peak pairs such as iso-orientin and orientin and quercetin-3-*O*-robinobioside and vitexin.

The final RP-HPLC method was validated in terms of specificity (correct peak identity and acceptable peak purity) by electrospray ionisation mass spectrometric (ESI-MS) detection. Retention times and UV-Vis, single stage MS and tandem MS (MS/MS) spectra were compared with those of authentic reference standards, where available, and literature data (Abad-Garcia *et al.*, 2008; Abad-García *et al.*, 2009; Iswaldi *et al.*, 2011; Beelders *et al.*, 2012b). The method was shown to be suitable for quantitative analysis of 16 compounds; linearity of the calibration curves was excellent (r^2 > 0.9999) and the method had good repeatability (RSD < 5.0%). Compound stability was also monitored over 24 h, with RSD values < 5.0% indicating no substantial degradation during the stability test. The developed method represents an improvement on that of Beelders *et al.*, (2012b) since the four eriodictyol-glucopyranosides could be quantified in addition to most of the other compounds separated in the previous method.

Traditionally rooibos is consumed as a herbal tea, colloquially known as rooibos tea, where the dried plant material is submerged in boiling water for approximately 5 min. Rooibos phenolic compounds can be efficiently extracted by use of hot water (Joubert *et al.*, 2013). However, not all rooibos phenolic compounds are extracted equally well in either polar aqueous water solutions or organic solutions, and by modifying solvent polarity with the addition an organic solvent (i.e. acetonitrile) the extraction potential can be increased. The optimal extraction solvent for rooibos plant material was evaluated using mixtures of acetonitrile (MeCN) and ethanol (EtOH) in water with sonication and heating steps. Extraction with 40% (v/v) MeCN provided the best overall phenolic extraction for green, semi-fermented and fermented rooibos plant material. These results are in agreement with previous studies, which showed that a 33% acetonitrile-water solvent composition provided

acceptable extraction of the majority of rooibos phenolic compounds (Manley *et al.*, 2006). Further experiments should be done to determine if the heating step is necessary and if sonication alone can provide sufficient extraction, thereby saving time and energy.

The RP-HPLC method was used to analyse rooibos water extracts of three different oxidative states, namely green, semi-fermented and fermented. As rooibos plants reproduce with seeds, genetic differences are commonly observed (Joubert & De Beer, 2011). To compensate for this, ten different rooibos bushes were harvested. The plant material of each bush was divided into three sub-batches, where each sub-batch was processed as either green, semi-fermented or fermented material. As the different treatments were applied to plant material from each bush, the quantitative changes of the rooibos phenolic compounds between treatments are as a result of the treatment and not genetic variation. Such a direct comparison of the effect of fermentation on phenolic composition of rooibos has not been previously performed.

The RP-HPLC-DAD method was used to separate and quantify the 16 target phenolic compounds in water extracts and 40% MeCN extracts prepared from the plant material. The water extracts are representative of common food ingredient extracts prepared from rooibos and would also give an indication of the phenolic composition of infusions. The 40% MeCN extracts represent maximum extraction from the plant material and served to characterise the phenolic content in the plant material. As 10 different plants were used for the quantitative analyses, the effect of genetic variation on phenolic content should be minimised. A general decrease in the majority of the phenolic compounds was observed during the rooibos oxidation process, except for the eriodictyol-glucopyranoside isomers and orientin, which are degradation products of aspalathin (Marais *et al.*, 2000). The levels of the eriodictyol-glucopyranoside isomers increased throughout the fermentation process. The concentration of orientin decreased from the green to semi-fermented oxidative state, but increased from the semi-fermented to fermented oxidative state, to the same level observed in the green oxidative state. Since the RP-HPLC-DAD method is suitable for the analysis of rooibos extracts of different oxidative states, the method can be used to monitor the phenolic composition of plant material sampled after different fermentation times. This can provide greater insight into the kinetics and temperature dependence of aspalathin oxidation during the fermentation process. Another application of the RP-HPLC-DAD method includes the possibility of monitoring the long term storage of rooibos extracts or other formulated products, where degradation of aspalathin to its oxidation products is expected depending on the storage conditions and time. This can provide valuable information on the conditions and formulations needed to prevent degradation of aspalathin. The developed RP-HPLC-DAD method is also suitable for biomedical studies, where the bioavailability or bioactivity of rooibos extracts is studied *in vitro* and *in vivo*.

Following the development of the quantitative RP-HPLC method, an off-line comprehensive HPCCC×LC method was designed. The first separation dimension (^{1}D) utilised a normal phase (NP) HPCCC gradient separation using an ethyl acetate-*n*-butanol-water (EBuWat) solvent system. Rooibos water extracts were previously found to form emulsions in a similar solvent system, resulting in loss of stationary phase or ruining of the column, therefore the ethanol insoluble fraction was removed prior to analysis (De Beer *et al.*, 2015).

The second separation dimension (^{2}D) was designed using an ultra high pressure LC (UHPLC) instrument instead of a conventional HPLC, as a rapid method with high efficiency was required to analyse a large number of ^{1}D fractions. The column selected for the ^{2}D method development was a Poroshell 120 SB C_{18} (100 x 2.1 mm i.d., 1.8 µm), which allowed a higher flow rate and efficiency than the ZORBAX SB C_{18} (100 x 2.1 mm i.d., 1.8 µm), which was also considered in preliminary experiments. The method gradient and flow rate was adapted from the quantitative HPLC method developed in the first part of this work to maximise performance (peak capacity) for shorter analysis times.

Optimisation of the off-line HPCCC×UHPLC method entailed consideration of the maximum peak capacity as a function of total analysis time for different fraction collection (sampling) times and ^{2}D cycle times (Kalili & De Villiers, 2013). Optimal conditions for a reasonable total analysis time were selected based on a sampling time of 1.25 min and ^{2}D cycle time of 20 min. The total practical peak capacity, determined by taking into account both the degree of under-sampling and orthogonality, was calculated as 3293. The total practical peak capacity obtained was relatively high compared to other studies using off-line LC×LC separation of phenolics, e.g. 2096 for rooibos (Beelders *et al.*, 2012a), 3137 and 2334, respectively, for cocoa and apple (analysis time >24 h) (Kalili & De Villiers, 2009) and between 2100 and 3000 for green tea (Kalili & De Villiers, 2010).

The comprehensive combination of HPCCC and RP-LC provided a highly orthogonal 2D method (orthogonality of ~80%, calculated using the average of conditional entropy and convex hull methods), which allowed the separation and tentative identification of 39 peaks, including the 16 compounds analysed using the developed quantitative HPLC method. The high degree of orthogonality can be attributed to the different separation mechanisms of the NP-HPCCC (liquid-liquid partition) and the RP-UHPLC (solid-liquid exchange) (Berthod *et al.*, 2009) modes. The 2D qualitative method compared well with that of Beelders *et al.*, (2012a), in terms of orthogonality with both giving values of ≥ 80% for off-line comprehensive 2D analysis. The high practical peak capacity is reflected in the excellent separation achieved by the HPCCC×UHPLC method.

The ^{2}D separation was also hyphenated to electrospray ionisation quadrupole time of flight (ESI-Q-TOF)-MS detection to obtain accurate mass single stage MS and MS/MS spectra in order to tentatively identify new compounds. The fractions were analysed only in positive ionisation mode due to limited instrument availability and since positive ionisation provides better structural elucidation information (Sakushima *et al.*, 1988; Abad-Garcia *et al.*, 2008). The remaining 23 unknown phenolic compounds were tentatively identified using a combination of UV-Vis and MS/MS data. Four of these were identified with the use of authentic reference standards: scolymoside, hesperidin, vicenin-2 and phloretin-3',5'-di-*C*-glucoside.

The presence of scolymoside, hesperidin and phloretin-3',5'-di-*C*-glucoside was reported for the first time here. These compounds were also previously detected in honeybush species (*Cyclopia* spp.) (De Beer *et al.*, 2012; Beelders *et al.*, 2014; Schulze *et al.*, 2014; Schulze *et al.*, 2016). Vicenin-2 and 3-hydroxyphloretin-di-*C*-hexoside were previously detected in both rooibos (Iswaldi *et al.*, 2011; Beelders *et al.*, 2012b) and honeybush (De Beer *et al.*, 2012; Schulze *et al.*, 2016). These compounds present in both *Aspalathus linearis* and *Cyclopia* spp, indicates a close chemotaxonomic relationship between these plants. Both honeybush and rooibos are from the same plant family, Fabaceae (Joubert *et al.*, 2008). This close relationship between the two plant species suggests a common ancestor, which explains why certain phenolic compounds associated with honeybush were detected in rooibos plant material, albeit at lower quantities

Many of the newly identified phenolic compounds were present in polar CCC fractions (the last fractions), and are weakly retained in RP-LC. Indeed, the pre-separation by CCC greatly facilitated the separation and tentative identification of these compounds (most believed to be aspalathin derivatives), which are normally poorly resolved by RP-LC. If, as seems likely based on the available data, these newly separated compounds are indeed derived from aspalathin, it would be prudent to develop an analytical method suitable for the quantification of the eriodictyol-glucopyranoside isomers, iso-orientin, orientin and aspalathin as well as these polar compounds, in order to determine the effect processing on their levels and their possible biological effects.

The tentative identification of the potentially new *C*-glycosides was not as successful, as these compounds have highly complex MS/MS spectra which hampered assignment of glycoside moieties and glycosylation positions. For the unambiguous assignment of these compounds, isolation and nuclear magnetic resonance (NMR) spectroscopy would be required. The isolation and purification of compounds present at low quantities in sufficient quantities for NMR analysis (2-50 mg) would likely be very challenging. Microfractionation combined with micro-NMR analyses may provide an alternative due to high sensitivity (Ghazani *et al.*, 2013; Wolfender *et al.*, 2015).

In conclusion, a quantitative HPLC-DAD method was successfully developed for the analysis of 16 rooibos phenolic compounds, including for the first time the eriodictyol-glucopyranoside isomers, oxidation products of aspalathin. The method is applicable to both organic and aqueous extracts. 40% MeCN was determined to provide the best overall extraction of rooibos phenolic compounds from green, semi-fermented and fermented plant material. The degradation process of aspalathin to the eriodictyol isomers, iso-orientin and orientin can now be studied in more detail using the developed RP-HPLC method, which may also prove useful in biological studies on *in vivo* degradation of rooibos phenolics. Analysis of green, semi-fermented and fermented plant material already provided evidence that the decrease in the aspalathin content of the plant material from green to fermented is accompanied by an increase in the content of the eriodictyol-glucopyranoside isomers. Furthermore, a qualitative HPCCC×RP-LC method was developed which allowed the separation and tentative identification of 39 rooibos phenolic compounds, 23 of which were not separated using the quantitative method. This was possible due to the high orthogonality obtained by the off-line combination of NP-HPCCC and RP-UHPLC separations. Future studies can investigate the effect different solvent systems on the NP-HPCCC separation. Preparative HPCCC separation should be implemented to allow higher sample injection, as phenolic compounds present at low quantities might not be detectable in the second dimension. Rooibos plant material can also be harvested from different geographical areas, which will allow for genetic and geographical variation to be represented. These samples can be pooled for 2D analyses with the goal of obtaining a pooled representative sample of rooibos phenolic content. Negative mode ESI-MS should also be applied in combination with 2-dimensional separation to enable detection of phenolic acids present in rooibos plant material (Sakushima *et al.*, 1988). Further studies can also be done to unambiguously identify the newly separated phenolic compounds using NMR or microNMR. Finally, biological studies can also be pursued to determine the biological properties of the newly identified compounds in conjunction with aspalathin or other major rooibos phenolic compounds. The qualitative 2D method, especially the first dimension, can be useful if applied to qualitative studies of other plant species (i.e. *Cyclopia* spp.), where multiple polar phenolic compounds still remain unknown due to the difficulty in separating polar compounds by RP-HPLC (De Beer *et al.*, 2012; Schulze *et al.*, 2016).

References

Abad-Garcia, B., Garmon-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2008). New features on the fragmentation and differentiation of *C*-glycosidic flavone isomers by

positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry,* **22**, 1834-1842.

Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B. & Vicente, F. (2009). Practical guidelines for characterization of *O*-diglycosyl flavonoid isomers by triple quadrupole MS and their applications for identification of some fruit juices flavonoids. *Journal of Mass Spectrometry,* **44**, 1017-1025.

Beelders, T., De Beer, D., Stander, M. A. & Joubert, E. (2014). Comprehensive phenolic profiling of *Cyclopia genistoides* (L.) Vent. by LC-DAD-MS and -MS/MS reveals novel xanthone and benzophenone constituents. *Molecules,* **19**, 11760-11790.

Beelders, T., Kalili, K. M., Joubert, E., De Beer, D. & De Villiers, A. (2012a). Comprehensive two-dimensional liquid chromatographic analysis of rooibos (*Aspalathus linearis*) phenolics. *Journal of Separation Science,* **35**, 1808-1820.

Beelders, T., Sigge, G. O., Joubert, E., De Beer, D. & De Villiers, A. (2012b). Kinetic optimisation of the reversed phase liquid chromatographic separation of rooibos tea (*Aspalathus linearis*) phenolics on conventional high performance liquid chromatographic instrumentation. *Journal of Chromatography A,* **1219**, 128-139.

Berthod, A., Maryutina, T., Spivakov, B., Shpigun, O. & Sutherland, I. A. (2009). Countercurrent chromatography in analytical chemistry (IUPAC technical report). *Pure and Applied Chemistry,* **81**, 355-387.

De Beer, D., Malherbe, C. J., Beelders, T., Willenburg, E. L., Brand, D. J. & Joubert, E. (2015). Isolation of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) using high-performance countercurrent chromatography: Sample loading and compound stability considerations. *Journal of Chromatography A,* **1381**, 29-36.

De Beer, D., Schulze, A., Joubert, E., De Villiers, A., Malherbe, C. & Stander, M. (2012). Food Ingredient extracts of *Cyclopia subternata* (Honeybush): variation in phenolic composition and antioxidant capacity. *Molecules,* **17**, 14602-14624.

Ghazani, A. A., McDermott, S., Pectasides, M., Sebas, M., Mino-Kenudson, M., Lee, H., Weissleder, R. & Castro, C. M. (2013). Comparison of select cancer biomarkers in human circulating and bulk tumor cells using magnetic nanoparticles and a miniaturized micro-NMR system. *Nanomedicine : Nanotechnology, Biology, and Medicine,* **9**, 1009-1017.

Hayes, R., Ahmed, A., Edge, T. & Zhang, H. (2014). Core-shell particles: preparation, fundamentals and applications in high performance liquid chromatography. *Journal of Chromatography A,* **1357**, 36-52.

Hübsch, Z., Van Vuuren, S. F. & Van Zyl, R. L. (2014). Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies? *South African Journal of Botany,* **93**, 148-156.

Iswaldi, I., Arraez-Roman, D., Rodriguez-Medina, I., Beltran-Debon, R., Joven, J., Segura-Carretero, A. & Fernandez-Gutierrez, A. (2011). Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Analytical and Bioanalytical Chemistry,* **400**, 3643-3654.

Joubert, E. & De Beer, D. (2011). Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany,* **77**, 869-886.

Joubert, E., De Beer, D., Malherbe, C. J., Muller, N., Bonnet, S. L., Van der Westhuizen, J. H. & Ferreira, D. (2013). Occurrence and sensory perception of *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in rooibos (*Aspalathus linearis*). *Food Chemistry,* **136**, 1078-1085.

Joubert, E., Gelderblom, W. C. A., Louw, A. & De Beer, D. (2008). South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides*—A review. *Journal of Ethnopharmacology,* **119**, 376-412.

Kalili, K. M. & De Villiers, A. (2009). Off-line comprehensive 2-dimensional hydrophilic interaction × reversed phase liquid chromatography analysis of procyanidins. *Journal of Chromatography A,* **1216**, 6274-6284.

Kalili, K. M. & De Villiers, A. (2010). Off-line comprehensive two-dimensional hydrophilic interaction x reversed phase liquid chromatographic analysis of green tea phenolics. *Journal of Separation Science,* **33**, 853-863.

Kalili, K. M. & De Villiers, A. (2013). Systematic optimisation and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography x reversed phase liquid chromatographic analysis of procyanidins, part I: Theoretical considerations. *Journal of Chromatography A,* **1289**, 58-68.

Kamakura, R., Son, M. J., De Beer, D., Joubert, E., Miura, Y. & Yagasaki, K. (2015). Antidiabetic effect of green rooibos (*Aspalathus linearis*) extract in cultured cells and type 2 diabetic model KK-A(y) mice. *Cytotechnology,* **67**, 699-710.

Kawano, A., Nakamura, H., Hata, S., Minakawa, M., Miura, Y. & Yagasaki, K. (2009). Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine,* **16**, 437-443.

Ku, S. K., Kwak, S., Kim, Y. & Bae, J. S. (2015). Aspalathin and nothofagin from rooibos (*Aspalathus linearis*) inhibits high glucose-induced inflammation *in vitro* and *in vivo*. *Inflammation,* **38**, 445-455.

Lee, W. & Bae, J. S. (2015). Anti-inflammatory effects of aspalathin and nothofagin from rooibos (*Aspalathus linearis*) *In Vitro* and *In Vivo*. *Inflammation,* **38**, 1502-1516.

Manley, M., Joubert, E. & Botha, M. (2006). Quantification of the major phenolic compounds, soluble solid content and total antioxidant activity of green rooibos (*Aspalathus*

linearis) by means of near infrared spectroscopy. *Journal of Near Infrared Spectroscopy,* **14**, 213-222.

Marais, C., Janse van Rensburg, W., Ferreira, D. & Steenkamp, J. A. (2000). (*S*)- and (*R*)-Eriodictyol-6-*C*-β-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*). *Phytochemistry,* **55**, 43-49.

Marnewick, J., Joubert, E., Joseph, S., Swanevelder, S., Swart, P. & Gelderblom, W. (2005). Inhibition of tumour promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas. *Cancer Letters,* **224**, 193-202.

Marnewick, J. L., Van der Westhuizen, F. H., Joubert, E., Swanevelder, S., Swart, P. & Gelderblom, W. C. (2009). Chemoprotective properties of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) herbal and green and black (*Camellia sinensis*) teas against cancer promotion induced by fumonisin B1 in rat liver. *Food and Chemical Toxicology,* **47**, 220-229.

Muller, C. J., Malherbe, C. J., Chellan, N., Yagasaki, K., Miura, Y. & Joubert, E. (2016). Potential of rooibos, its major *C*-glucosyl flavonoids and Z-2-(beta-D-glucopyranoloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. *Critical Reviews in Food Science and Nutrition,* DOI: 10.1080/10408398.2016.1157568.

Sakushima, A., Nishibe, S., Takeda, T. & Ogihara, Y. (1988). Positive and negative ion mass spectra of flavonoid glycosides by fast atom bombardment. *Journal of the Mass Spectrometry Society of Japan,* **36**, 71-80.

Schulze, A. E., De Beer, D., De Villiers, A., Manley, M. & Joubert, E. (2014). Chemometric analysis of chromatographic fingerprints shows potential of *Cyclopia maculata* (Andrews) Kies for production of standardized extracts with high xanthone content. *Journal of Agricultural and Food Chemistry,* **62**, 10542-10551.

Schulze, A. E., De Beer, D., Mazibuko, S. E., Muller, C. J., Roux, C., Willenburg, E. L., Nyunai, N., Louw, J., Manley, M. & Joubert, E. (2016). Assessing similarity analysis of chromatographic fingerprints of *Cyclopia subternata* extracts as potential screening tool for *in vitro* glucose utilisation. *Analytical and Bioanalytical Chemistry,* **408**, 639-649.

Shalliker, R. A. (2009). Two-dimensional HPLC analysis of oligostyrenes: comprehensive and online heart-cutting techniques. *Journal of Separation Science,* **32**, 2903-2911.

Van der Merwe, J. D., Joubert, E., Manley, M., De Beer, D., Malherbe, C. J. & Gelderblom, W. C. (2010). *In vitro* hepatic biotransformation of aspalathin and nothofagin, dihydrochalcones of rooibos (*Aspalathus linearis*), and assessment of metabolite antioxidant activity. *Journal of Agricultural and Food Chemistry,* **58**, 2214-2220.

Van der Merwe, J. D., Joubert, E., Richards, E. S., Manley, M., Snijman, P. W., Marnewick, J. L. & Gelderblom, W. C. (2006). A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. *Mutation Research,* **611**, 42-53.

Wolfender, J. L., Marti, G., Thomas, A. & Bertrand, S. (2015). Current approaches and challenges for the metabolite profiling of complex natural extracts. *Journal of Chromatography A,* **1382**, 136-164.

ADDENDUM A

Supplementary results pertaining to

Chapter 3

Table A.1 Characteristics of calibration curves obtained for HPLC analysis of phenolic standards

Compound	Number of calibration points	Wavelength (nm)	Linearity range (µg on-column)	Regression equation [a]	Correlation coefficient (r^2)
Eriodictyol-7-*O*-glucoside	8	288	0.0138-1.3762	$y = 2275.6\,x - 1.5209$	0.9999
Iso-orientin	8	350	0.0138-1.3800	$y = 2628\,x + 0.9578$	0.9999
Orientin	8	350	0.0133-1.3342	$y = 2620.3\,x - 2.5846$	0.9999
Aspalathin	9	288	0.0107-2.6627	$y = 2289.2\,x - 3.3236$	0.9999
Vitexin	8	350	0.0092-0.9248	$y = 2317.5\,x + 1.1062$	0.9999
Hyperoside	8	350	0.0094-0.9422	$y = 1688\,x - 6.3933$	0.9998
Rutin	8	350	0.0095-0.9511	$y = 1449.1\,x + 0.3963$	0.9999
Iso-vitexin	8	350	0.0094-0.9422	$y = 2439.2\,x - 1.7947$	0.9999
Iso-quercitrin	8	350	0.0092-0.9199	$y = 1750.4\,x - 2.3983$	0.9997
Luteolin-7-*O*-glucoside	8	350	0.0095-0.9461	$y = 2527.8\,x - 7.2660$	0.9999

[a] y = analyte response (peak area in mAU) and x = amount of standard compound injected (µg).

Table A.2 Percentage relative standard deviation (% RSD) of peak areas for the determination of analyte stability and analytical repeatability of polyphenolic compounds present in standard calibration mix, green and fermented samples

Peak nr	Compound	Stability	Repeatability
		24 h (n = 7)	(n = 6)
	Standard Calibration Mixture [a]		
na [b]	Eriodictyol-7-*O*-glucoside	0.97	0.38
6	Iso-orientin	0.62	0.19
7	Orientin	1.94	0.39
8	Aspalathin	0.59	0.18
10	Vitexin	0.57	0.28
11	Hyperoside	0.43	0.27
12	Rutin	0.38	0.56
13	Isovitexin	0.55	0.37
14	Isoquercitrin	0.47	0.53
15	Luteolin-7-*O*-glucoside	1.41	0.44
	Green Extract		
1	(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	1.71	1.34
2	(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	2.42	4.68
3	(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside	0.40	0.36
4	(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside	4.43	3.26
5	*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)	0.45	0.70
6	Iso-orientin	0.51	0.20
7	Orientin	0.47	0.27
8	Aspalathin	0.45	1.05
9	Quercetin-3-*O*-robinobioside	2.74	0.60
10	Vitexin	1.62	1.05
11	Hyperoside	3.34	2.49
12	Rutin	1.79	3.34
13	Isovitexin	0.52	1.42
14	Isoquercitrin	3.82	1.63
15	Luteolin-7-*O*-glucopyranoside	2.74	4.05
16	Nothofagin	0.43	0.10
	Fermented Extract		
1	(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.84	0.27
2	(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	0.50	2.28
3	(*S*)-eriodictyol-8-*C*-β-D-glucopyranoside	1.24	1.53
4	(*R*)-eriodictyol-8-*C*-β-D-glucopyranoside	1.71	5.26
5	*Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG)	0.55	0.72
6	Iso-orientin	0.95	2.21
7	Orientin	0.42	0.59
8	Aspalathin	0.78	1.80
9	Quercetin-3-*O*-robinobioside	3.60	1.13
10	Vitexin	2.43	2.08
11	Hyperoside	0.79	5.02
12	Rutin	1.79	2.70
13	Isovitexin	0.50	2.10
14	Isoquercitrin	1.89	1.72
15	Luteolin-7-*O*-glucopyranoside	2.05	3.67
16	Nothofagin	2.87	4.28

[a] µg injected on the column for each standard compound in calibration mix: eriodictyol-7-*O*-glucoside = 0.5505, iso-orientin = 0.5520, orientin = 0.5337, aspalathin = 1.0651, vitexin = 0.3699, hyperoside = 0.3769, rutin = 0.3804, Isovitexin = 0.3769, isoquercitrin = 0.3680, luteolin-7-*O*-glucoside = 0.3784.

[b] Standard compound not detected in sample, only used to quantify corresponding eriodictyol-glucopyranoside isomers.

Table A.3 Phenolic composition (mean ± standard deviation)[a] of green rooibos plant material with different extraction solvents

Extraction solvents	1	2	3	4	5	6	7	8	9	10	11	12	13+14	15	16
Distilled water	0,012 ± 0.001$_{C}$	0,012 ± 0.001$_{B}$	0,011 ± 0.001$_{EF}$	0,006 ± 0.002$_{EF}$	0,081 ± 0.006$_{CD}$	0,478 ± 0.162$_{D}$	0,064 ± 0.007$_{E}$	0,058 ± 0.006$_{D}$	0,055 ± 0.007$_{E}$	0,039 ± 0.002$_{E}$	0,007 ± 0.001$_{E}$	0,003 ± 0.000$_{FG}$	0,014 ± 0.001$_{D}$	0,015 ± 0.001$_{E}$	0,006 ± 0.000$_{DE}$
20% EtOH	0,011 ± 0.001$_{C}$	0,010 ± 0.001$_{C}$	0,015 ± 0.002$_{CD}$	0,010 ± 0.001$_{BC}$	0,096 ± 0.018$_{C}$	1,046 ± 0.188$_{BC}$	0,088 ± 0.016B$_{CD}$	0,085 ± 0.012$_{C}$	0,094 ± 0.015$_{CD}$	0,052 ± 0.010$_{CD}$	0,011 ± 0.002$_{D}$	0,004 ± 0.001$_{E}$	0,019 ± 0.003$_{BC}$	0,018 ± 0.002$_{E}$	0,007 ± 0.000$_{CD}$
40% EtOH	0,005 ± 0.001$_{E}$	0,004 ± 0.001$_{E}$	0,007 ± 0.001$_{G}$	0,005 ± 0.001$_{F}$	0,043 ± 0.004$_{E}$	0,451 ± 0.073$_{D}$	0,038 ± 0.006$_{F}$	0,039 ± 0.006$_{E}$	0,043 ± 0.007$_{EF}$	0,023 ± 0.004$_{F}$	0,005 ± 0.001$_{E}$	0,002 ± 0.000$_{G}$	0,008 ± 0.001$_{E}$	0,009 ± 0.001$_{F}$	0,005 ± 0.000$_{E}$
60% EtOH	0,006 ± 0.001$_{E}$	0,004 ± 0.001$_{E}$	0,010 ± 0.002$_{EF}$	0,007 ± 0.001$_{EF}$	0,076 ± 0.017$_{D}$	0,850 ± 0.180$_{C}$	0,071 ± 0.015$_{DE}$	0,078 ± 0.017$_{C}$	0,084 ± 0.017$_{D}$	0,044 ± 0.009$_{DE}$	0,010 ± 0.002$_{D}$	0,004 ± 0.001$_{EF}$	0,016 ± 0.003$_{CD}$	0,017 ± 0.003$_{E}$	0,008 ± 0.002$_{BC}$
80% EtOH	0,008 ± 0.001$_{D}$	0,015 ± 0.001$_{A}$	0,017 ± 0.002$_{BC}$	0,009 ± 0.001$_{CD}$	0,153 ± 0.011$_{A}$	1,654 ± 0.059$_{A}$	0,147 ± 0.010$_{A}$	0,165 ± 0.010$_{A}$	0,173 ± 0.013$_{A}$	0,092 ± 0.005$_{A}$	0,020 ± 0.001$_{A}$	0,009 ± 0.001$_{B}$	0,033 ± 0.002$_{A}$	0,036 ± 0.002$_{B}$	0,016 ± 0.001$_{A}$
100% EtOH	0,005 ± 0.001$_{E}$	0,011 ± 0.000$_{BC}$	0,012 ± 0.001$_{DE}$	0,008 ± 0.000$_{DE}$	0,087 ± 0.003$_{CD}$	1,230 ± 0.093$_{B}$	0,107 ± 0.009$_{B}$	0,103 ± 0.008$_{B}$	0,111 ± 0.010$_{BC}$	0,063 ± 0.005$_{B}$	0,014 ± 0.001$_{C}$	0,006 ± 0.001$_{CD}$	0,020 ± 0.002$_{B}$	0,024 ± 0.002$_{D}$	0,009 ± 0.001$_{B}$
20% MeCN	0,014 ± 0.001$_{B}$	0,011 ± 0.002$_{BC}$	0,014 ± 0.002$_{D}$	0,010 ± 0.001$_{C}$	0,093 ± 0.008$_{C}$	0,946 ± 0.117$_{C}$	0,085 ± 0.011$_{CD}$	0,088 ± 0.012$_{BC}$	0,077 ± 0.009$_{D}$	0,054 ± 0.007$_{BC}$	0,010 ± 0.001$_{D}$	0,005 ± 0.001$_{DE}$	0,020 ± 0.003$_{B}$	0,023 ± 0.003$_{D}$	0,009 ± 0.001$_{B}$
40% MeCN	0,016 ± 0.001$_{A}$	0,012 ± 0.001$_{B}$	0,021 ± 0.001$_{A}$	0,015 ± 0.001$_{A}$	0,135 ± 0.003$_{B}$	1,513 ± 0.024$_{A}$	0,139 ± 0.004$_{A}$	0,151 ± 0.005$_{A}$	0,126 ± 0.005$_{B}$	0,088 ± 0.005$_{A}$	0,018 ± 0.001$_{AB}$	0,010 ± 0.001$_{A}$	0,032 ± 0.002$_{A}$	0,040 ± 0.002$_{A}$	0,016 ± 0.002$_{A}$
60% MeCN	0,011 ± 0.001$_{C}$	0,007 ± 0.000$_{E}$	0,018 ± 0.001$_{B}$	0,012 ± 0.001$_{B}$	0,129 ± 0.002$_{B}$	1,505 ± 0.000$_{A}$	0,135 ± 0.002$_{A}$	0,150 ± 0.001$_{A}$	0,125 ± 0.001$_{B}$	0,086 ± 0.001$_{A}$	0,017 ± 0.000$_{B}$	0,009 ± 0.000$_{AB}$	0,031 ± 0.000$_{A}$	0,040 ± 0.001$_{A}$	0,015 ± 0.001$_{A}$
80% MeCN	0,005 ± 0.001$_{E}$	0,012 ± 0.001$_{B}$	0,009 ± 0.001$_{F}$	0,006 ± 0.001$_{EF}$	0,092 ± 0.007$_{CD}$	1,025 ± 0.054$_{BC}$	0,091 ± 0.004$_{BC}$	0,103 ± 0.007$_{B}$	0,091 ± 0.006$_{D}$	0,058 ± 0.003$_{BC}$	0,012 ± 0.001$_{CD}$	0,006 ± 0.000$_{C}$	0,021 ± 0.001$_{B}$	0,028 ± 0.002$_{C}$	0,014 ± 0.001$_{A}$
100% MeCN	nq[b]	nq[b]	nq[b]	nq[b]	0,025 ± 0.008$_{F}$	0,491 ± 0.139$_{D}$	0,053 ± 0.015$_{EF}$	0,029 ± 0.008$_{E}$	0,028 ± 0.009$_{F}$	0,015 ± 0.004$_{F}$	0,005 ± 0.002$_{E}$	0,003 ± 0.000$_{G}$	0,005 ± 0.001$_{E}$	0,011 ± 0.003$_{F}$	0,006 ± 0.001$_{DE}$

[a] Mean content values (g.100 g plant material^{-1}); [b] Quantified as eriodictyol-7-*O*-glucopyranoside equivalents; [c] Quantified as rutin equivalents.
Different subscript capital letters indicate significant (P<0.05) differences between treatments.
Abbreviations: EtOH, ethanol; MeCN, acetonitrile; numbers (*cf.* Table 3).

Table A.4 Phenolic composition (mean ± standard deviation)[a] of semi-fermented rooibos plant material with different extraction solvents

Extraction solvents	1	2	3	4	5	6	7	8	9	10	11	12	13+14	15	16
Distilled water	0,013 ± 0.001$_{D}$	0,013 ± 0.001$_{D}$	0,008 ± 0.001$_{D}$	0,004 ± 0.000C$_{F}$	0,051 ± 0.003$_{CD}$	0,339 ± 0.025$_{E}$	0,023 ± 0.002$_{F}$	0,035 ± 0.003$_{EF}$	0,034 ± 0.003$_{E}$	0,024 ± 0.002$_{E}$	0,004 ± 0.000$_{E}$	0,002 ± 0.000$_{F}$	0,009 ± 0.001$_{E}$	0,009 ± 0.001$_{F}$	0,009 ± 0.001$_{F}$
20% EtOH	0,021 ± 0.001$_{A}$	0,020 ± 0.001$_{A}$	0,014 ± 0.002$_{A}$	0,009 ± 0.001$_{B}$	0,086 ± 0.008$_{A}$	0,714 ± 0.056A$_{B}$	0,049 ± 0.003$_{BC}$	0,079 ± 0.008$_{C}$	0,090 ± 0.007$_{A}$	0,045 ± 0.004$_{BC}$	0,010 ± 0.001$_{B}$	0,003 ± 0.000$_{DE}$	0,018 ± 0.002$_{BC}$	0,018 ± 0.002$_{DE}$	0,018 ± 0.001$_{CD}$
40% EtOH	0,018 ± 0.001$_{BC}$	0,017 ± 0.002$_{BC}$	0,014 ± 0.001$_{A}$	0,010 ± 0.001$_{A}$	0,084 ± 0.009$_{A}$	0,732 ± 0.076A$_{B}$	0,050 ± 0.005$_{ABC}$	0,086 ± 0.008$_{BC}$	0,093 ± 0.008$_{A}$	0,047 ± 0.005$_{BC}$	0,010 ± 0.009$_{B}$	0,004 ± 0.000$_{D}$	0,018 ± 0.002$_{BC}$	0,018 ± 0.001$_{D}$	0,018 ± 0.001$_{C}$
60% EtOH	0,008 ± 0.001$_{F}$	0,007 ± 0.001$_{EF}$	0,006 ± 0.001$_{E}$	0,005 ± 0.001$_{EF}$	0,042 ± 0.007$_{D}$	0,347 ± 0.059$_{E}$	0,023 ± 0.004$_{F}$	0,044 ± 0.007$_{E}$	0,047 ± 0.007$_{D}$	0,022 ± 0.004$_{E}$	0,005 ± 0.001$_{E}$	0,002 ± 0.000$_{F}$	0,009 ± 0.001$_{E}$	0,009 ± 0.001$_{F}$	0,009 ± 0.001$_{DEF}$
80% EtOH	0,007 ± 0.001$_{FG}$	0,009 ± 0.001$_{E}$	0,006 ± 0.001$_{E}$	0,004 ± 0.001$_{F}$	0,040 ± 0.004$_{D}$	0,350 ± 0.039$_{E}$	0,022 ± 0.002$_{F}$	0,043 ± 0.003$_{E}$	0,047 ± 0.004$_{D}$	0,022 ± 0.003$_{E}$	0,005 ± 0.001$_{E}$	0,002 ± 0.000$_{F}$	0,008 ± 0.001$_{E}$	0,008 ± 0.001$_{F}$	0,008 ± 0.000$_{EF}$
100% EtOH	0,010 ± 0.001$_{E}$	0,009 ± 0.001$_{E}$	0,009 ± 0.001$_{BC}$	0,006 ± 0.000$_{DE}$	0,053 ± 0.006$_{C}$	0,601 ± 0.066$_{C}$	0,040 ± 0.005$_{DE}$	0,065 ± 0.008$_{D}$	0,070 ± 0.009$_{C}$	0,036 ± 0.004$_{D}$	0,009 ± 0.001$_{C}$	0,003 ± 0.000$_{DE}$	0,013 ± 0.002$_{D}$	0,013 ± 0.002$_{E}$	0,013 ± 0.001$_{CDE}$
20% MeCN	0,016 ± 0.002$_{C}$	0,016 ± 0.002$_{C}$	0,011 ± 0.002$_{B}$	0,008 ± 0.001$_{AC}$	0,062 ± 0.01$_{BC}$	0,472 ± 0.011$_{D}$	0,034 ± 0.009$_{E}$	0,060 ± 0.011$_{D}$	0,055 ± 0.012$_{D}$	0,034 ± 0.008$_{D}$	0,007 ± 0.001$_{D}$	0,003 ± 0.000$_{E}$	0,013 ± 0.003$_{D}$	0,013 ± 0.003$_{DE}$	0,013 ± 0.001$_{C}$
40% MeCN	0,020 ± 0.001$_{AB}$	0,019 ± 0.001$_{AB}$	0,015 ± 0.001$_{A}$	0,010 ± 0.001$_{A}$	0,083 ± 0.005$_{A}$	0,717 ± 0.057$_{AB}$	0,051 ± 0.003$_{AB}$	0,093 ± 0.006$_{B}$	0,083 ± 0.006$_{AB}$	0,051 ± 0.003$_{AB}$	0,010 ± 0.001$_{B}$	0,005 ± 0.000$_{B}$	0,020 ± 0.001$_{AB}$	0,020 ± 0.001$_{B}$	0,020 ± 0.001$_{B}$
60% MeCN	0,019 ± 0.000$_{AB}$	0,017 ± 0.000B$_{C}$	0,015 ± 0.000$_{A}$	0,011 ± 0.001$_{A}$	0,092 ± 0.000$_{A}$	0,800 ± 0.021$_{A}$	0,056 ± 0.000$_{A}$	0,106 ± 0.002$_{A}$	0,093 ± 0.001$_{A}$	0,057 ± 0.001$_{A}$	0,012 ± 0.000$_{A}$	0,005 ± 0.000$_{A}$	0,022 ± 0.000$_{A}$	0,022 ± 0.000$_{A}$	0,022 ± 0.001$_{A}$
80% MeCN	0,016 ± 0.016$_{C}$	0,016 ± 0.002$_{C}$	0,009 ± 0.001$_{C}$	0,006 ± 0.001$_{D}$	0,071 ± 0.007$_{B}$	0,643 ± 0.040$_{BC}$	0,044 ± 0.004$_{CD}$	0,080 ± 0.008$_{C}$	0,073 ± 0.007B$_{C}$	0,044 ± 0.004$_{C}$	0,009 ± 0.001$_{BC}$	0,004 ± 0.000$_{C}$	0,016 ± 0.001$_{C}$	0,016 ± 0.001$_{C}$	0,016 ± 0.002$_{B}$
100% MeCN	0,005 ± 0.005$_{G}$	0,005 ± 0.000$_{F}$	0,003 ± 0.000$_{G}$	0,002 ± 0.000$_{G}$	0,023 ± 0.002$_{E}$	0,319 ± 0.036$_{E}$	0,027 ± 0.002$_{F}$	0,024 ± 0.003$_{F}$	0,025 ± 0.002$_{E}$	0,012 ± 0.001$_{F}$	0,005 ± 0.000$_{E}$	0,002 ± 0.000$_{F}$	0,005 ± 0.000$_{F}$	0,005 ± 0.001$_{F}$	0,005 ± 0.000$_{G}$

[a] Mean content values (g.100 g plant material^{-1}); [b] Quantified as eriodictyol-7-*O*-glucopyranoside equivalents; [c] Quantified as rutin equivalents.
Different subscript capital letters indicate significant (P<0.05) differences between treatments.
Abbreviations: EtOH, ethanol; MeCN, acetonitrile; numbers (*cf.* Table 3).

Table A.5 Phenolic composition (mean ± standard deviation)[a] of fermented rooibos plant material with different extraction solvents

Extraction solvents	1	2	3	4	5	6	7	8	9	10	11	12	13+14	15	16
Distilled water	0,019 ± 0.005$_{D}$	0,020 ± 0,005$_{C}$	0,007 ± 0.002$_{C}$	0,006 ± 0.002$_{B}$	0,047 ± 0.011$_{CDE}$	0,092 ± 0.021$_{D}$	0,007 ± 0.002$_{E}$	0,027 ± 0.006$_{FG}$	0,027 ± 0.006$_{EF}$	0,020 ± 0.004$_{E}$	0,004 ± 0.001$_{DE}$	0,002 ± 0.000$_{CD}$	0,007 ± 0.001$_{EF}$	0,007 ± 0.001$_{BC}$	0,004 ± 0.001$_{E}$
20% EtOH	0,028 ± 0.005$_{B}$	0,030 ± 0,005$_{AB}$	0,011 ± 0.002$_{AB}$	0,010 ± 0.002$_{A}$	0,061 ±0.010$_{AB}$	0,150 ± 0.032$_{BC}$	0,013 ± 0.003$_{B}$	0,046 ± 0.011$_{ED}$	0,055 ± 0.013$_{B}$	0,028 ± 0.006$_{CD}$	0,007 ± 0.001$_{B}$	0,003 ± 0.001$_{BC}$	0,009 ± 0.002$_{CDE}$	0,010 ± 0.002$_{B}$	0,006 ± 0.001$_{CD}$
40% EtOH	0,020 ± 0.004$_{D}$	0,021 ± 0,004$_{C}$	0,008 ± 0.001$_{C}$	0,007 ± 0.001$_{B}$	0,044 ± 0.010$_{CDE}$	0,114 ± 0.025$_{CD}$	0,009 ± 0.002$_{CDE}$	0,037 ± 0.007$_{EF}$	0,043 ± 0.008$_{CD}$	0,020 ± 0.005$_{E}$	0,005 ± 0.001$_{CD}$	0,002 ± 0.000$_{CD}$	0,006 ± 0.002$_{F}$	0,008 ± 0.001$_{BC}$	0,005 ± 0.000$_{DE}$
60% EtOH	0,024 ± 0.001$_{BCD}$	0,025 ± 0,002$_{BC}$	0,009 ± 0.001$_{BC}$	0,008 ± 0.004$_{B}$	0,051 ± 0.004$_{BCD}$	0,143 ± 0.012$_{BC}$	0,011 ± 0.001$_{BCD}$	0,048 ± 0.004$_{CDE}$	0,055 ± 0.004$_{BC}$	0,026 ± 0.002$_{CDE}$	0,007 ± 0.001$_{BC}$	0,003 ± 0.000$_{BC}$	0,008 ± 0.001$_{DEF}$	0,010 ± 0.001$_{B}$	0,006 ± 0.000$_{BC}$
80% EtOH	0,036 ± 0.004$_{A}$	0,035 ± 0.004$_{A}$	0,012 ± 0.002$_{A}$	0,010 ± 0.002$_{A}$	0,073 ± 0.009$_{A}$	0,231 ± 0.028$_{A}$	0,017 ± 0.002$_{A}$	0,077 ± 0.009$_{A}$	0,084 ± 0.010$_{A}$	0,043 ± 0.005$_{A}$	0,010 ± 0.001$_{A}$	0,004 ± 0.000$_{A}$	0,012 ± 0.001$_{A}$	0,016 ± 0.002$_{A}$	0,009 ± 0.001$_{A}$
100% EtOH	0,020 ± 0.001$_{CD}$	0,022 ± 0.002$_{C}$	0,008 ± 0.001$_{C}$	0,007 ± 0.001$_{B}$	0,036 ± 0.002$_{E}$	0,139 ± 0.006$_{BC}$	0,011 ± 0.001$_{BCD}$	0,043 ± 0.003$_{ED}$	0,048 ± 0.004$_{BC}$	0,024 ± 0.002$_{DE}$	0,006 ± 0.001$_{BC}$	0,003 ± 0.000$_{B}$	0,007 ± 0.000$_{DEF}$	0,009 ± 0.000$_{B}$	0,007 ± 0.000$_{B}$
20% MeCN	0,020 ± 0.005$_{D}$	0,021 ± 0.005$_{C}$	0,007 ± 0.002$_{C}$	0,007 ± 0.002$_{B}$	0,040 ± 0.010$_{DE}$	0,102 ± 0.024$_{D}$	0,008 ± 0.003$_{DE}$	0,036 ± 0.008$_{EF}$	0,033 ± 0.008$_{DE}$	0,022 ± 0.005$_{DE}$	0,004 ± 0.001$_{DE}$	0,002 ± 0.000$_{BCD}$	0,007 ± 0.001$_{EF}$	0,009 ± 0.002$_{B}$	0,005 ± 0.001$_{CDE}$
40% MeCN	0,030 ± 0.002$_{AB}$	0,032 ± 0.002$_{AB}$	0,011 ± 0.001$_{AB}$	0,010 ± 0.001$_{A}$	0,063 ± 0.004$_{AB}$	0,170 ± 0.009$_{B}$	0,014 ± 0.001$_{B}$	0,064 ± 0.003$_{B}$	0,055 ± 0.003$_{B}$	0,038 ± 0.002$_{AB}$	0,008 ± 0.001$_{B}$	0,005 ± 0.001$_{A}$	0,012 ± 0.001$_{AB}$	0,017 ± 0.001$_{A}$	0,009 ± 0.001$_{A}$
60% MeCN	0,026 ± 0.003$_{BC}$	0,027 ± 0.003$_{BC}$	0,010 ± 0.001$_{ABC}$	0,008 ± 0.001$_{AB}$	0,054 ± 0.007$_{BC}$	0,154 ± 0.020$_{B}$	0,111 ± 0.002$_{BCD}$	0,059 ± 0.009$_{BC}$	0,050 ± 0.006$_{BC}$	0,034 ± 0.005$_{BC}$	0,007 ± 0.001$_{B}$	0,004 ± 0.006$_{A}$	0,010 ± 0.002$_{BC}$	0,015 ± 0.002$_{A}$	0,009 ± 0.001$_{A}$
80% MeCN	0,027 ± 0.005$_{B}$	0,027 ± 0.004$_{BC}$	0,008 ± 0.001$_{C}$	0,007 ± 0.001$_{B}$	0,050 ± 0.008$_{CD}$	0,154 ± 0.024$_{B}$	0,011 ± 0.002$_{BC}$	0,055 ± 0.010$_{BCD}$	0,048 ± 0.008$_{BC}$	0,032 ± 0.006$_{BC}$	0,007 ± 0.001$_{B}$	0,004 ± 0.008$_{A}$	0,009 ± 0.002$_{CD}$	0,015 ± 0.003$_{A}$	0,008 ± 0.001$_{A}$
100% MeCN	0,011 ± 0.002$_{E}$	0,011 ± 0.002$_{D}$	0,004 ± 0.001$_{D}$	0,003 ± 0.001$_{C}$	0,016 ± 0.003$_{F}$	0,080 ± 0.016$_{D}$	0,008 ± 0.002$_{E}$	0,016 ± 0.002$_{G}$	0,015 ± 0.002$_{F}$	0,007 ± 0.001$_{F}$	0,003 ± 0.001$_{E}$	0,002 ± 0.000$_{D}$	0,003 ± 0.001$_{G}$	0,006 ± 0.001$_{C}$	0,006 ± .0001$_{CD}$

[a] Mean content values (g.100 g plant material^{-1}); [b] Quantified as eriodictyol-7-*O*-glucopyranoside equivalents; [c] Quantified as rutin equivalents.
Different subscript capital letters indicate significant (P<0.05) differences between treatments.
Abbreviations: EtOH, ethanol; MeCN, acetonitrile; numbers (*cf.* Table 3).

ADDENDUM B

Supplementary results pertaining to

Chapter 4

B.1.

```
yaxis = [1:48]*1.25;
xaxis = Time(:,1);
matrixData = MyData(:,4:4:end);
X= Time ;
Y= yaxis ;
Z=  matrixData;
matrix2D = Z;
matrix2D = matrix2D(500:14000,9:end);
matrix2D = matrix2D>2.00;
total = sum(sum(matrix2D));
sizeX = size(matrix2D);
sizeY = sizeX(1);
sizeX = sizeX(2);
numYcom = floor(sizeY/sizeX);
squareMatrix = zeros(sizeX);
for jth = 0:sizeX-1
   for ith = 0:sizeX-1
      for nth = 0:numYcom-1
         squareMatrix(jth+1,ith+1)=
squareMatrix(jth+1,ith+1)+matrix2D(numYcom*jth+nth+1,ith+1);
      end
   end
end
Px = sum(squareMatrix)/total;
Xsize=size(Px);
Xsize=Xsize(2);
Py = sum(squareMatrix')/total;
Ysize=size(Py);
Ysize=Ysize(2);
PxDif = repmat(Px,Ysize,1);
PyDif = repmat(Py',1,Xsize);
pMatrix2D = squareMatrix/total;
matrixXdif=pMatrix2D./PxDif;
matrixYdif=pMatrix2D./PyDif;
matrixXdif=log2(matrixXdif);
matrixYdif=log2(matrixYdif);
matrixXdif(isnan(matrixXdif)) = 0;
matrixYdif(isnan(matrixYdif)) = 0;
matrixXdif(isinf(matrixXdif)) = 0;
matrixYdif(isinf(matrixYdif)) = 0;
HxGy = - sum(sum( pMatrix2D.*matrixYdif ))
HyGx = - sum(sum( pMatrix2D.*matrixXdif ))
Hx = Px.*log2(Px);
Hy = Py.*log2(Py);
Hx(isnan(Hx)) = 0;
Hy(isnan(Hy)) = 0;
Hx = -sum(Hx)
Hy = -sum(Hy)
O1 = HxGy/Hx
O2 = HyGx/Hy
Hxy = (squareMatrix/total).*log2( (squareMatrix/total) );
Hxy(isnan(Hxy)) = 0;
Hxy = -sum(sum( Hxy ))
```

```
HxGy = Hxy - Hy
HyGx = Hxy - Hx
O1 = HxGy/Hx*100
O2 = HyGx/Hy*100
```

B.2.

```
function A = mConvexHull2D( )
[stmfile, stmpath] = uigetfile({'*.xls;*xlsx'}, 'Select excel file');
h=figure('Position',[500,500,400,100],'MenuBar','none','NumberTitle','off');
htext = uicontrol(h,'Style','text','String','Click the OK button then go on to select the
normalized 1D values.','Position',[10,50,380,40]);
b1= uicontrol(h,'Style','pushbutton','String','OK',
'Position',[165,20,70,20],'Callback','uiresume(gcbf)');
uiwait(gcf);
close(h);
t = xlsread(fullfile(stmpath, stmfile), -1);
rawx=t(:,1);
h=figure('Position',[500,500,400,100],'MenuBar','none','NumberTitle','off');
htext = uicontrol(h,'Style','text','String','Click the OK button then go on to select the
normalized 2D values.','Position',[10,50,380,40]);
b1= uicontrol(h,'Style','pushbutton','String','OK',
'Position',[165,20,70,20],'Callback','uiresume(gcbf)');
uiwait(gcf);
close(h);
t = xlsread(fullfile(stmpath, stmfile), -1);
rawy=t(:,1);
% Convex Hull Area Script
K=convhull(rawx,rawy);
figure
plot(rawx,rawy,'x')
hold
plot(rawx(K),rawy(K),'r-')
areaconvhull=polyarea(rawx(K),rawy(K));
msgbox(num2str(areaconvhull));
end
```